

NOVEL POLYKETIDE DERIVATIVES AND RECOMBINANT  
METHODS FOR MAKING SAME

This application is a continuation-in-part of co-pending U.S. application Serial No. 07/642,734, filed January 17, 1991.

Technical Field

The present invention relates to novel polynucleotide sequences, proteins encoded therefrom which are involved in the biosynthesis of polyketides, methods for directing the biosynthesis of novel polyketides using those polynucleotide sequences and novel derivatives produced therefrom. In particular, the invention relates to the production of novel polyketide derivatives through manipulation of the genes encoding polyketide synthases.

Background of the Invention

Polyketides are a large class of natural products that includes many important antibiotic, antifungal, anticancer, antihelminthic, and immunosuppressant compounds such as erythromycins, tetracyclines, amphotericins, daunorubicins, avermectins, and rapamycins. Their synthesis proceeds by an ordered condensation of acyl esters to generate carbon chains of varying length and substitution pattern that are later converted to mature polyketides. This process has long been recognized as resembling fatty acid biosynthesis, but with important differences. Unlike a fatty acid synthase, a typical polyketide synthase is programmed to make many choices during carbon chain assembly: for example, the choice of "starter" and "extender" units, which are often selected from acetate, propionate or butyrate residues in a defined sequence by the polyketide synthase. The choice of using a full cycle of reduction-dehydration-reduction after some condensation steps, omitting it completely, or using one of two incomplete cycles (reduction alone or reduction followed by dehydration) is additionally programmed, and determines the pattern of keto or hydroxyl groups and the degree of saturation at different points in the chain. Finally, the stereochemistry for the substituents at many of the carbon atoms is programmed by the polyketide synthase.

*Streptomyces* and the closely related *Saccharopolyspora* genera are producers of a prodigious diversity of polyketide metabolites. Because of the commercial significance of these compounds, a great amount of effort has been expended in the study of *Streptomyces* and *Saccharopolyspora* genetics. Consequently, much is known about these organisms and several cloning vectors and techniques exist for their transformation.

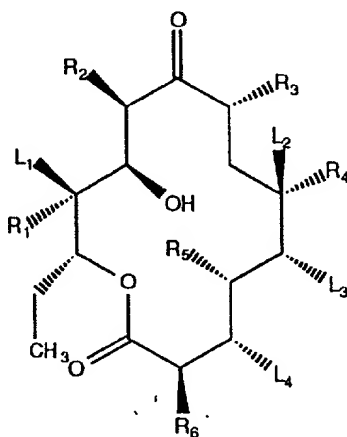
Although many polyketides have been identified, there remains the need to obtain novel polyketide structures with enhanced properties. Current methods of obtaining such molecules include screening of natural isolates and chemical modification of existing polyketides, both of which are costly and time consuming. Current screening methods are based on gross properties of the molecules, i.e. antibacterial, antifungal activity, etc., and both

*a priori* knowledge of the structure of the molecules obtained or predetermination of enhanced properties are virtually impossible. Chemical modification of preexisting structures has been successfully employed to obtain novel polyketides, but still suffers from practical limitations to the type of compounds obtainable, largely connected to the poor yield of multistep synthesis and available chemistry to effect modifications. Modifications which are particularly difficult to achieve are those involving additions or deletions of carbon side chains. Accordingly, there exists a considerable need to obtain molecules wherein such changes can be specified and performed in a cost effective manner and with high yield.

The present invention solves these problems by providing reagents (specifically, polynucleotides, vectors comprising the polynucleotides and host cells comprising the vectors) and methods to generate novel polyketides by *de novo* biosynthesis rather than by chemical modification.

### Summary of the Invention

In one aspect, the present invention provides compounds of the formula:



X

wherein R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub>, R<sub>4</sub>, R<sub>5</sub>, and R<sub>6</sub> are independently selected from Q wherein Q is selected from the group consisting of (a) -H, (b) -Me, (c) -Et, and (d) -OH; L<sub>1</sub> and L<sub>2</sub> are independently -H or -OH; L<sub>3</sub> is D-desosamine or -OH; and L<sub>4</sub> is L-mycarose, L-cladinose or -OH with the proviso that when R<sub>1</sub>-R<sub>5</sub> are -Me, R<sub>6</sub> is other than -H or -Me.

Preferred compounds of the invention are those in which Q is selected from the group consisting of (a), (b) and (c) above or (a), (b) and (d) above or (a), (c) and (d) above or (b), (c) and (d) above or (a) and (b) above or (a) and (c) above or (a) and (d) above or (b) and (c) above or (c) and (d) above and L<sub>1</sub>, L<sub>2</sub>, L<sub>3</sub> and L<sub>4</sub> are as defined above. Other preferred compounds include those in which R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub>, R<sub>4</sub>, R<sub>5</sub> and R<sub>6</sub> are all -H or -Et or -OH and L<sub>1</sub>, L<sub>2</sub>, L<sub>3</sub> and L<sub>4</sub> are as defined above. Still other preferred compounds include didesmethyl, tridesmethyl, tetradesmethyl, pentadesmethyl and hexadesmethyl derivatives of the compounds of formula

X and particularly, di- tri-, tetra-, penta- and hexadesmethyl derivatives of erythromycins A and B. Other especially preferred compounds of formula X include 6,10-didesmethyl-6-ethylerythromycin A, 10,12-didesmethyl-12-deoxy-12-ethylerythromycin A, 10,12-didesmethyl-12-deoxy-10-hydroxyerythromycin A, 6,10,12-tridesmethyl-6,12-diethylerythromycin A, 6,10,12-tridesmethyl-6-deoxy-6,12-diethylerythromycin A, 10-desmethylerythronolide B, 10-desmethyl-6-deoxyerythronolide B, 12-desmethylerythronolide B, 12-desmethyl-6-deoxyerythronolide B, 12-desmethyl-12-ethylerythronolide B, 6-desmethyl-6-deoxy-6-ethylerythronolide B, 10-desmethylerythromycin A, 10-desmethyl-12-deoxyerythromycin A, 10-desmethyl-6,12-dideoxyerythromycin A, 12-desmethylerythromycin A, 12-desmethyl-12-deoxyerythromycin A, 12-desmethyl-6,12-dideoxyerythromycin A, 6-desmethyl-6-ethylerythromycin A, 12-desmethyl-12-ethylerythromycin A, 12-desmethyl-12-deoxy-12-ethylerythromycin A, 10-desmethyl-10-hydroxyerythromycin A, 12-desmethyl-12-epihydroxyerythromycin A, 10,12-didesmethylerythromycin A, 10,12-didesmethyl-12-deoxyerythromycin A, 10,12-didesmethyl-6,12-dideoxyerythromycin A, 10-desmethylerythronolide B, 10-desmethyl-6-deoxyerythronolide B, 12-desmethylerythronolide B, 12-desmethyl-6-deoxyerythronolide B, 10-desmethylerythromycin A, 10-desmethyl-12-deoxyerythromycin A, 10-desmethyl-6,12-dideoxyerythromycin A, 12-desmethylerythromycin A, 12-desmethyl-12-deoxyerythromycin A, 12-desmethyl-6,12-dideoxyerythromycin A, 10,12-didesmethylerythromycin A, 10,12-didesmethyl-12-deoxyerythromycin A, and 10,12-didesmethyl-6,12-dideoxyerythromycin A. Most preferred compounds include 10-desmethylerythromycin A, 10-desmethyl-12-deoxyerythromycin A, and 12-desmethyl-12-deoxyerythromycin A.

In another aspect, the present invention provides an isolated polynucleotide sequence or fragment thereof which encodes an enzymatically active acyltransferase domain from a PKS selected from *Streptomyces hygroscopicus*, *Streptomyces venezuelae*, and *Streptomyces caelestis*. Preferably, the polynucleotide sequence is SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:29 or SEQ ID NO:30. In another preferred embodiment, the polynucleotide sequence encodes an acyltransferase domain selected from the group consisting of SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33 and SEQ ID NO:34.

The present invention also provides a vector comprising a polynucleotide sequence or fragment thereof which encodes an enzymatically active acyltransferase domain from *Streptomyces*. Preferably, the polynucleotide sequence is selected from those described above and the *Streptomyces* is *Streptomyces hygroscopicus*, *Streptomyces venezuelae*, or *Streptomyces caelestis*. A particularly preferred vector is pCS5. Other vectors of the invention include pUC18/LigAT2, pEryAT1/LigAT2, pEryAT2/LigAT2, pUC18/venAT, pEryAT1/venAT, pUC19/rapAT14, pEryAT1/rapAT14, pEryAT2/rapAT14, pUC/5'-flank/ethAT, pUC/ethAT/C-6, pEAT4, pUC18/NidAT6, and pEryAT2/NidAT6.

In another aspect, the invention provides host cells transformed with a vector as described above. The host cell may be a bacterial cell and preferably is selected from the group consisting of *E. coli* and *Bacillus* species. Alternatively, the host cell is a polyketide-producing microorganism. A preferred polyketide-producing host cell is selected from the group consisting of *Saccharopolyspora* species, *Nocardia* species, *Micromonospora* species, *Arthrobacter* species, *Streptomyces* species, *Actinomadura* species, and *Dactylosporangium* species. An even more preferred polyketide-producing host cell is selected from the group consisting of *Saccharopolyspora hirsuta*, *Micromonospora rosaria*, *Micromonospora megalomicea*, *Streptomyces antibioticus*, *Streptomyces mycarofaciens*, *Streptomyces avermitilis*, *Streptomyces hygroscopicus*, *Streptomyces caelestis*, *Streptomyces tsukubaensis*, *Streptomyces fradiae*, *Streptomyces platensis*, *Streptomyces violaceoniger*, *Streptomyces ambofaciens*, *Streptomyces griseoplanus*, and *Streptomyces venezuelae*. Of these host cells, *Saccharopolyspora erythraea*, *Streptomyces hygroscopicus*, *Streptomyces venezuelae*, and *Streptomyces caelestis* are most preferred.

The invention also provides a method for altering the substrate specificity of a polyketide synthase in a first polyketide-producing microorganism comprising the steps of (a) isolating a first and second genomic DNA segment, each comprising a polyketide synthase wherein the first genomic DNA segment is from the first polyketide-producing microorganism and the second genomic DNA segment is from the first polyketide-producing microorganism or a second polyketide-producing microorganism;

(b) identifying one or more discrete fragments of the first genomic DNA segment, each of which encodes an acyltransferase domain;

(c) identifying one or more discrete fragments of the second genomic DNA segment, each of which encodes a related domain to the acyltransferase domain of the first genomic DNA segment; and

(d) transforming a cell of the first polyketide-producing microorganism with one or more of the fragments from step (c) under conditions suitable for the occurrence of a homologous recombination event, leading to the replacement of one or more of the fragments from the first genomic DNA segment with one or more of the fragments from step (c). In one embodiment, the first polyketide-producing microorganism is *Saccharopolyspora erythraea* and the second polyketide-producing microorganism is *Streptomyces*. Preferred *Streptomyces* are selected from the group consisting of *Streptomyces antibioticus*, *Streptomyces mycarofaciens*, *Streptomyces avermitilis*, *Streptomyces hygroscopicus*, *Streptomyces caelestis*, *Streptomyces tsukubaensis*, *Streptomyces fradiae*, *Streptomyces platensis*, *Streptomyces violaceoniger*, *Streptomyces ambofaciens*, and *Streptomyces venezuelae*. Even more preferred *Streptomyces* are *Streptomyces caelestis*, *Streptomyces hygroscopicus*, or *Streptomyces venezuelae*. In a second embodiment, the first polyketide-producing microorganism is a *Streptomyces* as described above and the second polyketide-



producing microorganism is *Saccharopolyspora erythraea*. Also in a preferred embodiment, the related domain is selected from the group consisting of SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, and SEQ ID NO:34.

#### Brief Description of the Drawings

The present invention will be more readily appreciated in connection with the accompanying drawings.

FIG. 1 is a proposed metabolic pathway for the biosynthesis of erythromycin A in *Sac. erythraea*.

FIG. 2 is a schematic representation of the erythromycin PKS.

FIG. 3 is a Growtree analysis of AT domains from *Streptomyces hygroscopicus* (*S. hygroscopicus*; LigAT2 and rapAT1-14), *Streptomyces venezuelae* (*S. venezuelae*; venAT) and *Saccharopolyspora erythraea* (*Sac. erythraea*; eryAT1-6).

FIG. 4a is a schematic representation of gene replacements of EryAT1 with LigAT2 or venAT and EryAT2 with LigAT2 in *Sac. erythraea*.

FIG. 4b is a schematic representation of gene replacements of EryAT4 with an ethyl AT (NidAT5) in *Sac. erythraea*.

FIG. 5 is a diagrammatic representation of gene replacement by homologous recombination.

FIG. 6 is a schematic representation of the genetic organization of the Ligase-PKS cluster from *S. hygroscopicus* ATCC 29253.

FIG. 7 represents the nucleotide sequence (SEQ ID NO:1, top strand) and corresponding amino acid sequence (SEQ ID NO:31, bottom strand) of LigAT, the malonyl AT domain from module 2 of the Ligase-PKS cluster of *S. hygroscopicus* ATCC 29253.

FIG. 8 is a diagrammatic representation of the strategy to clone the LigAT2 domain.

FIG. 9 is a flow diagram depicting the cloning of the EryAT1 flanking regions in plasmid pCS5.

FIG. 10 is a flow diagram depicting construction of pEryAT1/LigAT2.

FIG. 11 is a computer generated PhosphorImage of a Southern analysis of chromosomal DNA from *Sac. erythraea* ER720 EryAT1/LigAT2 resolvants cut with *SphI* and probed with an approximately 3 kb *EcoRI/HindIII* fragment from pEryAT1/LigAT2. As shown in lanes 3, 4 and 7 the probe hybridized with fragments of 3.5 and 1.6 kb, indicating that LigAT2 had replaced EryAT1 in the chromosomes of these resolvants (clone #10, #11 and #24 respectively). Lanes 5 and 6: chromosomal DNA from *Sac. erythraea* ER720 resolvants to wild-type (wt); lanes 1 and 9: molecular weight markers (1 kb ladder).

FIG. 12 is a computer reproduction of a TLC plate on which the products produced by *Sac. erythraea* ER720 EryAT1/LigAT2 were run. Lanes 1 and 7: erythromycin A standard (5 µg); lanes 2 and 6: compounds produced by wild-type *Sac. erythraea* ER720; lanes 3, 4 and

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5: compounds produced by *Sac. erythraea* ER720 EryAT1/LigAT2 resolvants to mutant-type, clones #10, #11 and #24 respectively.

FIG. 13 is a flow diagram depicting the cloning of the EryAT2 flanking regions in plasmid pCS5.

FIG. 14 is a flow diagram depicting construction of pEryAT2/LigAT2.

FIG. 15 is a computer generated PhosphorImage of a Southern analysis of chromosomal DNA from *Sac. erythraea* ER720 EryAT2/LigAT2, cut with *SphI* and probed with an approximately 1 kb LigAT2 sequence. As seen in lane 3, an approximately 900 base pair fragment hybridized with the probe, indicating that LigAT2 had replaced EryAT2 in this resolvant. Lane 2: chromosomal DNA from wild-type (wt) *Sac. erythraea* ER720; lanes 1 and 5: molecular weight markers (1 kb ladder).

FIG. 16 is a computer reproduction of a TLC plate on which the products produced by *Sac. erythraea* ER720 EryAT2/LigAT2 were run. Lanes 1 and 6: erythromycin B standard (5 µg); lanes 2 and 5: erythromycin A standard (5 µg); lane 4: compounds produced by wild-type *Sac. erythraea* ER720; lane 3: compounds produced by *Sac. erythraea* ER720 EryAT2/LigAT2 resolvant, clone #2-4.

FIG. 17 is a computer reproduction of a Xerox image of a bioautography plate of products made by *Sac. erythraea* ER720 EryAT2/LigAT2 against *S. aureus*. Lanes 1 and 7: erythromycin B standard (1 µg); lanes 2 and 6: erythromycin A standard; lane 3: compounds produced by wild-type *Sac. erythraea* ER720; lane 4: extract from an 0.1 mL culture of *Sac. erythraea* ER720 EryAT2/LigAT2 resolvant clone #2-4; lane 5: extract from an 0.5 mL culture of *Sac. erythraea* ER720 EryAT2/LigAT2 resolvant clone #2-4.

FIG. 18 represents the nucleotide sequence (SEQ ID NO:2, top strand) and corresponding amino acid sequence (SEQ ID NO:32, bottom strand) of venAT, the malonate AT domain from the PKS cluster (hereinafter designated pven4) from *S. venezuelae* ATCC 15439.

FIG. 19 is a diagrammatic representation of the strategy to clone the venAT domain.

FIG. 20 is a flow diagram depicting construction of pEryAT1/venAT.

FIG. 21 is a computer generated PhosphorImage of a Southern analysis of chromosomal DNA from *Sac. erythraea* ER720 EryAT1/venAT resolvants, cut with *PvuII* and probed with a venAT sequence. As seen in lanes 4 and 5, the probe hybridized with fragments of 4.2 and 2.4 kb, indicating that venAT had replaced EryAT1 in these resolvants. Lane 1: molecular weight markers (1 kb ladder); lane 2: chromosomal DNA from a wild-type *Sac. erythraea* ER720; lane 3: chromosomal DNA from a *Sac. erythraea* ER720 EryAT1/venAT integrant; lane 4: chromosomal DNA from *Sac. erythraea* ER720 EryAT1/venAT resolvant clone #C.1; lane 5: chromosomal DNA from *Sac. erythraea* ER720 EryAT1/venAT resolvant clone #C.4.

FIG. 22 is a computer reproduction of a TLC plate on which the products produced by *Sac. erythraea* ER720 EryAT1/venAT were run. Lane 1: erythromycin A standard (EryA; 5 µg) and 3-α-mycarosylerythronolide B (MEB; 10 µg); lane 2: compounds produced by *Sac. erythraea* ER720 EryAT1/venAT resolvant clone #C.4.

FIG. 23 is a diagrammatic representation of the strategy to clone the rapAT14 domain.

FIG. 24 is a flow diagram depicting construction of pEryAT1/rapAT14.

FIG. 25 is a computer generated PhosphorImage of a Southern analysis of chromosomal DNA from an *Sac. erythraea* ER720 EryAT1/rapAT14 resolvant, cut with *SpyI* and probed with an *EcoRI-HindIII* fragment from pCS5AT1-flank. As shown in lane 2 the probe hybridized with a 1.6 kb fragment indicating that rapAT14 had replaced EryAT1 in the chromosome of this resolvant. Lane 1: molecular weight markers (1 kb ladder); lane 2: chromosomal DNA from *Sac. erythraea* ER720 EryAT1/rapAT14 resolvant clone #4-A(1); lane 3: chromosomal DNA from wild-type *Sac. erythraea* ER720.

FIG. 26 is a computer reproduction of a TLC plate on which the products produced by *Sac. erythraea* ER720 EryAT1/rapAT14 were run. Lane 1: erythromycin A standard (5 µg); lane 2: compounds produced by *Sac. erythraea* ER720 EryAT1/rapAT14 resolvant.

FIG. 27 is a flow diagram depicting construction of pEryAT2/rapAT14.

FIG. 28 is a computer generated PhosphorImage of a Southern analysis of chromosomal DNA from *Sac. erythraea* ER720 EryAT2/rapAT14 resolvants, cut with *BspI* and probed with a fragment of 5'-flanking region of eryAT2. As shown in lanes 5, 6 and 7, the probe hybridized with a 4.3 kb fragment, indicating that rapAT14 had replaced EryAT2 in the chromosomes of these resolvants. Lane 1: molecular weight markers (1 kb ladder); lane 2: chromosomal DNA from wild-type *Sac. erythraea* ER720; lane 3: chromosomal DNA from *Sac. erythraea* ER720 resolvant to wild-type, clone #1.1; lane 4: chromosomal DNA from a *Sac. erythraea* ER720 EryAT2/rapAT14 integrant; lanes 5-7: chromosomal DNA from *Sac. erythraea* ER720 EryAT2/rapAT14 resolvant clones #1.2, #1.3 and #1.4 respectively.

FIG. 29 is a computer reproduction of a TLC plate on which the products produced by *Sac. erythraea* ER720 EryAT2/rapAT14 were run. Lane 1: erythromycin A and erythronolide B (EryA and EB, respectively; 5 µg each); lane 2: compounds produced by wild-type *Sac. erythraea* ER720; lanes 3-5: compounds produced by *Sac. erythraea* ER720 EryAT2/rapAT14 resolvant clones #1.2, #1.3 and #1.4 respectively.

FIG. 30 is a computer reproduction of a bioassay of compounds made by *Sac. erythraea* ER720 EryAT2/rapAT14 resolvant clones #1.2, #1.3 and #1.4 and resolvant to wild-type clone #1.1.

FIG. 31 is a computer generated PhosphorImage of a Southern analysis of a cosmid DNA library constructed from *Streptomyces caelestis* NRRL-2821 chromosomal DNA.

Lanes 1-19: DNA prepared from 19 clones, digested with *Sst*I and probed with a *Streptomyces caelestis* NRRL-2821 PKS specific probe.

FIG. 32 is a schematic representation of the genetic organization of the PKS cluster from *Streptomyces caelestis* NRRL-2821.

FIG. 33 is a diagram of the structure of the macrolide ring of niddamycin.

FIG. 34 represents the nucleotide sequence (SEQ ID NO:29, top strand) and corresponding amino acid sequence (SEQ ID NO:33, bottom strand) of NidAT5, the ethyl AT domain from module 5 of the PKS cluster of *Streptomyces caelestis* NRRL-2821.

FIG. 35 is a flow diagram depicting the construction of pUC/ethAT/C-6.

FIG. 36 is a diagram showing the nucleotide changes made to create an *Avr*II site at the 5' end of NidAT5.

FIG. 37 is a diagram of the replacement plasmid pEAT4.

FIG. 38 is a computer generated PhosphorImage of a Southern analysis of chromosomal DNA from *Sac. erythraea* ER720 EAT4 resolvants digested with *Mlu*I and probed with a 900 bp DNA fragment spanning a KS/AT domain in *Streptomyces caelestis* NRRL-2821. Lane assignments are as follows: 1) wild type ER720; 2-7) resolvant clones. The resolvants with the NidAT5 domain in place of EryAT4 produced a strongly hybridizing 1.8 kb fragment (lanes 4, 5, and 7) which is missing in clones which resolved back to wild type (lanes 2, 3, and 6).

FIG. 39 is a computer reproduction of a TLC plate showing the products made by *Sac. erythraea* EAT4-46 after growing in SCM or SCM + 50 mM butyric acid.

FIG. 40 is a computer generated PhosphorImage of a Southern analysis of clones from a cosmid DNA library constructed from *Streptomyces caelestis* NRRL-2821 chromosomal DNA. Clones were digested with *Sma*I and probed with a 900 bp DNA fragment spanning a KS/AT domain in *Streptomyces caelestis* NRRL-2821.

FIG. 41 represents the nucleotide sequence (SEQ ID NO:30, top strand) and corresponding amino acid sequence (SEQ ID NO:34, bottom strand) of NidAT6, the AT domain in module 6 of the niddamycin PKS cluster.

FIG. 42 is a diagrammatic representation of the strategy to clone the NidAT6 domain.

FIG. 43 is a flow diagram depicting construction of pEryAT2/NidAT6.

## DETAILED DESCRIPTION OF THE INVENTION

### I. Definitions:

For the purposes of the present invention as disclosed and claimed herein, the following terms are defined:

The term "polyketide" as used herein refers to a large and diverse class of natural products including but not limited to antibiotic, anticancer, antihelminthic, antifungal,

pigment, and immunosuppressant compounds. Antibiotics include but are not limited to anthracyclines, tetracyclines, polyethers, polyenes, ansamycins, and macrolides of various types such as avermectins, erythromycins, and niddamycins. The term polyketide is also intended to refer to compounds of this class that can be used as intermediates in chemical syntheses. For example, erythromycin A is a polyketide that is isolated and used in the synthesis of the antibiotic clarithromycin. Polyketides used as intermediates do not themselves necessarily have any biological or therapeutic activity.

The term "polyketide-producing microorganism" as used herein includes but is not limited to bacteria from the order *Actinomycetales*, *Myxococcales* or other *Eubacteriales* that can produce a polyketide. Examples of actinomycetes and myxobacteria that produce polyketides include but are not limited to *Saccharopolyspora erythraea*, *Saccharopolyspora hirsuta*, *Micromonospora rosaria*, *Micromonospora megalomicea*, *Sorangium cellulosum*, *Streptomyces antibioticus*, *Streptomyces mycarofaciens*, *Streptomyces avermitilis*, *Streptomyces hygroscopicus*, *Streptomyces caelestis*, *Streptomyces tsukubaensis*, *Streptomyces fradiae*, *Streptomyces platensis*, *Streptomyces violaceoniger*, *Streptomyces ambofaciens*, *Streptomyces venezuelae* and various other *Streptomyces*, *Actinomadura*, *Dactylosporangium* and *Amycolotopsis* strains that produce polyketides. Yeast and fungi that produce polyketides are also considered "polyketide-producing microorganisms". Examples of fungi that produce polyketides include but are not limited to members of the genus *Aspergillus*.

The term "polyketide synthase" (PKS) as used herein refers to a complex of enzyme activities responsible for the biosynthesis of polyketides. The enzymatic activities contained within a PKS include but are not limited to  $\beta$ -ketoreductase (KR), dehydratase (DH), enoylreductase (ER),  $\beta$ -ketoacyl ACP synthase (KS), acyl carrier protein (ACP), acyltransferase (AT) and thioesterase (TE). The polypeptide fragment responsible for each enzymatic activity is referred to as a "domain". A "module" refers to a group or set of domains which carry out one condensation step in the process of polyketide formation and may or may not include domains which effect processing of the  $\beta$ -carbonyl group in the growing polyketide.

The term "Type I PKS" as used herein refers to a PKS which is a large multifunctional protein and is exemplified by DEBS (see below). The term "Type II PKS" refers to a PKS having several separate, largely monofunctional enzymes, and is exemplified by the PKSs responsible for the biosynthesis of actinorhodin and tetracenomycin (C.R. Hutchinson and I. Fujii, *Annu. Rev. Microbiol.* **49**: 201-238 (1995)).

The term "cognate domains" as used herein refers to the members of a specific set of domains which constitute a naturally occurring single module.

The term "related domain" or "heterologous domain" as used herein refers to a PKS domain which is functionally similar to a second PKS domain. By "functionally similar" it is

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meant that each domain catalyzes a particular type of reaction but acts upon a different substrate. For example, the AT domain of module 1 of *Sac. erythraea* (eryAT1) and the AT domain of module 14 of *S. hygroscopicus* (rapAT14) both catalyze the transfer of an extender unit to a corresponding ACP domain. In the case of *Sac. erythraea*, however, eryAT1 utilizes methylmalonyl CoA as a substrate whereas in *S. hygroscopicus*, rapAT14 utilizes malonyl CoA. Thus, eryAT1 and rapAT14 are considered to be "related" or "heterologous" domains.

The term "condensation" as used herein refers to the addition of an extender unit to the nascent polyketide chain and requires the action of KS, AT and ACP domains of the PKS.

The term "starter" as used herein refers to a coenzyme A thioester of a carboxylic acid which is used by a polyketide synthase as the first building block of the polyketide.

The term "extender" as used herein refers to a coenzyme A thioester of a dicarboxylic acid that is incorporated into a polyketide by a polyketide synthase at positions other than the first position.

The term "DEBS" as used herein refers to the enzyme 6-deoxyerythronolide B synthase, the PKS that builds the polyketide-derived macrolactone 6-deoxyerythronolide B (6-DEB).

The term "*eryA*" as used herein refers to the genes which encode the DEBS.

The term "homologous recombination" as used herein refers to crossing over between DNA strands containing identical sequences.

The term "isolated" as used herein means that the material is removed from its original environment (e.g. the natural environment where the material is naturally occurring). For example, a naturally occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, which is separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that the vector or composition is not part of the natural environment.

The term "restriction fragment" as used herein refers to any linear DNA generated by the action of one or more restriction enzymes.

The term "transformation" as used herein refers to the introduction of DNA into a recipient microorganism, irrespective of the method used for the insertion into the microorganism.

The term "replicon" as used herein means any genetic element, such as a plasmid, chromosome or virus, that behaves as an autonomous unit of polynucleotide replication within a cell. A "vector" is a replicon in which another polynucleotide fragment is attached, such as to bring about the replication and/or expression of the attached fragment.

The terms "recombinant polynucleotide" or "recombinant polypeptide" as used herein means at least a polynucleotide or polypeptide which by virtue of its origin or manipulation is

not associated with all or a portion of the polynucleotide or polypeptide with which it is associated in nature and/or is linked to a polynucleotide or polypeptide other than that to which it is linked in nature.

The term "host cell" as used herein, refers to both prokaryotic and eukaryotic cells which are used as recipients of the recombinant polynucleotides and vectors provided herein.

The term "open reading frame" or "ORF" as used herein refers to a region of a polynucleotide sequence which encodes a polypeptide; this region may represent a portion of a coding sequence or a total coding sequence.

## II. The Invention

In its broadest sense, the present invention entails novel polyketides with therapeutic activity (e.g. antimicrobial, anticancer, antifungal, immunosuppressant and/or antihelminthic activity) and immediate compounds of such polyketides. The invention also provides a method for producing novel polyketides *in vivo* by selectively altering the genetic information of an organism that naturally produces a polyketide. The present invention further provides isolated and purified polynucleotides that encode PKS domains (i.e. polypeptides) from polyketide-producing microorganisms, fragments thereof, vectors containing those polynucleotides, and host cells transformed with those vectors. These polynucleotides, fragments thereof, and vectors comprising the polynucleotides can be used as reagents in the above described method. Portions of the polynucleotide sequences disclosed herein are also useful as primers for the amplification of DNA or as probes to identify related domains from other polyketide-producing microorganisms.

## III. Polynucleotides

The present invention provides isolated and purified polynucleotides that encode PKS domains (i.e. polypeptides) and fragments thereof which are involved in the production of polyketides. Polynucleotides included within the scope of the invention may be in the form of RNA, DNA, cDNA, genomic DNA and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single-stranded may be the coding (sense) strand or non-coding (anti-sense) strand. The coding sequence which encodes a polypeptide may be identical to a coding sequence provided herein or may be a different coding sequence which, as a result of the redundancy or degeneracy of the genetic code, encodes the same polypeptide as the DNA provided herein.

Polynucleotides may include only the coding sequence for a particular polypeptide or for a polypeptide which is functionally equivalent to the polypeptide sequences provided herein. Additionally, the invention includes variant polynucleotides containing modifications such as polynucleotide deletions, substitutions or additions; and any polypeptide modification resulting from the variant polynucleotide sequence. A polynucleotide of the present

invention also may have a coding sequence which is a naturally occurring allelic variant of the coding sequence provided herein.

Probes and primers constructed according to the polynucleotide sequences provided herein are also contemplated as within the scope of the present invention and can be used in various methods to provide various types of analysis. For example, primer sequences may be designed according to polynucleotide sequences which encode particular domains and then used to amplify polynucleotide sequences of the same or other related domains using well-known amplification techniques such as the polymerase chain reaction (PCR) and the ligase chain reaction (LCR). (PCR has been disclosed in U.S. patents 4,683,195 and 4,683,202, and LCR, in EP-A- 320 308 to K. Backman published June 16, 1989 and EP-A-439 182 to K. Backman *et al.*, published July 31, 1991, all of which are incorporated herein by reference). Generation of primers for use in other amplification techniques or in variations of these amplification techniques, (such as nested PCR) is also contemplated within the scope of the invention and is considered within the knowledge of the routine practitioner.

Probes and primers may be designed from conserved nucleotide regions of a polynucleotide of interest or from non-conserved nucleotide regions of a polynucleotide of interest. Generally, nucleic acid probes are developed from non-conserved or unique regions when maximum specificity is desired, and nucleic acid probes are developed from conserved regions when assaying for nucleotide regions of related members of a multigene family or in related species. Probes can also be labeled with radioisotopes or other detection labels for screening of recombinant libraries.

Various methods for synthesizing primers and probes are well-known in the art as are methods for attaching labels to primers or probes. For example, it is a matter of routine to synthesize desired nucleic acid primers or probes using conventional nucleotide phosphoramidite chemistry and instruments available from Applied Biosystems, Inc., (Foster City, CA), Dupont (Wilmington, DE), or Milligen (Bedford MA). Many methods have been described for labeling oligonucleotides such as the primers or probes of the present invention. Commercially available probe labeling kits include those from Amersham Life Science (Arlington Heights, IL), Promega (Madison, WI), Enzo Biochemical (New York, NY) and Clontech (Palo Alto, CA).

#### IV. Vectors and Host Cells

The present invention provides vectors which include polynucleotides of the present invention and host cells which are genetically engineered with vectors of the present invention.

##### a. Vectors and Expression Systems

The present invention includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid



or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences from prokaryotic or eukaryotic sources. Large numbers of suitable plasmids and vectors are known to those of skill in the art, and are commercially available. Vectors which are particularly useful for cloning and expression in intermediate hosts include but are not limited to: (a) Bacterial: pBR322 (ATCC 37017); pGEM (Promega Biotec, Madison, WI), pUC, pSPORT1 and pProEx1 (Life Technologies, Gaithersburg, MD); pQE70, pQE60, pQE-9 (Qiagen); pBs, phagescript, psiX174, pBluescript SK, pBsKS, pNH8a, pNH16a, pNH18a, pNH46a (Stratagene®, La Jolla, CA); pTrec99A, pKK223-3, pKK233-3, pDR540, pRIT5, and pGEX4T (Pharmacia®, Piscataway, NJ); and (b) Eukaryotic: pWLneo, pSV2cat, pOG44, pXT1, pSG (Stratagene®); pSVK3, pBPV, pMSG, pSVL (Pharmacia®); pcDNA3.1 (Invitrogen, Carlsbad, CA). Other appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Maniatis *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Press, N.Y., 1982), which is hereby incorporated by reference. Generally however, any plasmid or vector may be used as long as it is replicable and viable in a host.

In another embodiment, the construct is an expression vector which also comprises regulatory sequences operably linked to the sequence of interest, to direct mRNA synthesis and polypeptide production. Regulatory sequences known to operate in prokaryotic and/or eukaryotic cells include inducible and non-inducible promoters for regulating mRNA transcription, ribosome binding sites for translation initiation, stop codons for translation termination and transcription terminators and/or polyadenylation signals. In addition, an expression vector may include appropriate sequences for amplifying expression.

Promoter regions may be selected from any desired gene. Particular named bacterial promoters include *lacZ*, *gpt*,  $\lambda$  P<sub>R</sub>,  $\lambda$  P<sub>L</sub>, *trc*, *trp*, *ermE* and its derivatives such as *ermE*PIΔTGG, also known in the art as *ermE*\*, (Bibb, M. J., *et al.*, *Molecular Microbiology*, **14**(3): 533-545 (1994)), *melCI*, and *actII* (C.M. Kao, *et al.*, *Science*, **265**: 509-512 (1994)). Eukaryotic promoters include cytomegalovirus (CMV) immediate early, herpes simplex virus (HSV) thymidine kinase, early and late SV40, LTRs from retroviruses, mouse metallothionein-I, prion protein and neuronal specific enolase (NSE). Selection of the appropriate promoter is well within the level of ordinary skill in the art. In addition, a recombinant expression vector will include an origin of replication and selectable marker (such as a gene conferring resistance to an antibiotic (eg. neomycin, chloramphenicol, ampicillin, or thiostrepton) or a reporter gene (eg. luciferase)) which permit selection of stably transformed or transfected host cells.

In any expression vector, a heterologous structural sequence (i.e. a polynucleotide of the present invention) is assembled in appropriate phase with translation initiation and termination sequences. Optionally, the heterologous sequence will encode a fusion protein

including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Eukaryotic expression vectors will also generally comprise an origin of replication, a suitable promoter operably linked to a sequence of interest and also any necessary translation enhancing sequence, polyadenylation site, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early promoter, enhancer, and polyadenylation sites may be used to provide the required genetic elements. Such vectors may also include an enhancer sequence to increase transcription of a gene. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription rate. Examples include the SV40 enhancer on the late side of the replication origin (bp 100 to 270), a cytomegalovirus early promoter enhancer, a polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

#### i. Vector construction

The appropriate DNA sequence may be inserted into a vector by a variety of procedures. Generally, site-specific DNA cleavage is performed by treating the DNA with suitable restriction enzymes under conditions which are generally specified by the manufacturer of these commercially available enzymes. Usually, about 1 microgram ( $\mu\text{g}$ ) of plasmid or DNA sequence is cleaved by 1 unit of enzyme in about 20 microliters ( $\mu\text{L}$ ) of buffer solution by incubation at  $37^\circ\text{C}$  for 1 to 2 hours. After incubation with the restriction enzyme, protein can be removed by phenol/chloroform extraction and the DNA recovered by precipitation with ethanol. The cleaved fragments may be separated using polyacrylamide or agarose gel electrophoresis, according to methods known by the routine practitioner. (See Maniatis *et al.*, *supra*).

Ligations are performed using standard buffer and temperature conditions and with a ligase (such as T4 DNA ligase) and ATP. Sticky end ligations require less ATP and less ligase than blunt end ligations. Vector fragments may be treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIAP) to remove the 5'-phosphate and thus prevent religation of the vector. Ligation mixtures are transformed into suitable cloning hosts such as *E. coli* and successful transformants selected by methods including antibiotic resistance, and then screened for the correct construct.

#### ii. Transformation/Transfection

Transformation or transfection of an appropriate host with a construct of the invention, such that the host produces recombinant polypeptides, may also be performed in a variety of ways. For example, a construct may be introduced into a host cell by calcium chloride or polyethylene glycol transformation, lithium chloride or calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation. These and other methods for transforming/transfecting host cells are well known to routine practitioners (see

L. Davis *et al.*, "Basic Methods in Molecular Biology", 2nd edition, Appleton and Lang, Paramount Publishing, East Norwalk, CT (1994) and D.A. Hopwood *et al.*, Genetic Manipulation of *Streptomyces*: a laboratory manual, The John Innes Foundation, Norwich, England (1985)).

#### 5           b. Host Cells

In one embodiment, the present invention provides host cells containing recombinant constructs as described below. In one aspect, a host cell may be an "intermediate" host which is used to produce polynucleotides of the invention on a large-scale basis (for the purpose of cloning and/or verifying recombinant polynucleotide sequences, for example) or as a means to maintain such polynucleotide sequences over time (i.e. as maintenance or storage strains). A "production" host is a host cell which is used to produce novel polyketides. The host cell (either intermediate or production) can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or a prokaryotic cell, such as a bacterial cell. Lower eukaryotic and prokaryotic cells are preferred intermediate and production hosts.

Representative examples of appropriate hosts include bacterial cells, such as *E. coli*, *Bacillus subtilis*, *Saccharopolyspora erythraea*, *Streptomyces caelestis*, *Streptomyces hygroscopicus*, *Streptomyces venezuelae*; and various other species within the genera *Arthrobacter*, *Micromonospora*, *Nocardia*, *Pseudomonas*, *Streptomyces*, *Staphylococcus*, and *Saccharopolyspora*, although others (of eukaryotic origin) may also be employed. Additional representative examples of host cells are polyketide-producing microorganisms (as defined above). The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings provided herein.

Host cells are genetically engineered (transduced, transformed, transfected, conjugated, or electroporated) with the vectors of this invention which may be a cloning vector or an expression vector. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants, or as a source of a biosynthetic substrate. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

#### 30           V. Novel Polyketides and Methods of Making Novel Polyketides

The invention also provides novel polyketides, intermediate compounds thereof, and methods for producing novel polyketides. The methods utilize the polyketide biosynthetic genes from *Sac. erythraea* (i.e. the *eryA* genes) as well as those from other known polyketide-producing microorganisms and/or putative polyketide-producing microorganisms (i.e. those having nucleotide sequences which hybridize to known PKS sequences but whose polyketide products are unknown).

The organization of *eryA* and the DEBS encoded therefrom (see FIG. 1 and FIG. 2) have been described in co-pending U.S. application Serial No. 07/642,734, filed January 17, 1991, which is incorporated herein by reference in its entirety. As FIG. 2 shows, DEBS is organized in modules, with each module being responsible for one condensation step through the action of the resident KS, AT and ACP domains within that module wherein an extender unit, methylmalonyl CoA, is added first to the starter unit, propionyl CoA, and then successively to the growing acyl chain. The precise succession of the elongation steps is dictated by the order of the six modules: module 1 determines the first condensation; module 2, the second; module 3, the third, and so on until the sixth condensation step has occurred. In addition, the choice of extender unit that is incorporated into a growing polyketide chain at each condensation is determined, in whole or in part, by the AT domain within each module. In the case of DEBS, the extender unit incorporated is always methylmalonate. Thus, as 6-deoxyerythronolide B grows through successive condensations, two carbons are added to the nascent chain and every other carbon, starting with the carbon corresponding to C-12 in the ring, carries a methyl group as a side chain.

As also seen in FIG. 2, the processing of the growing carbon chain after each condensation is determined by the information within each module. Thus,  $\beta$ -ketoreduction of the  $\beta$ -keto group generated by the condensation event takes place after each condensation step except the third, as determined by the presence of an active KR domain in each module except module 3, whereas dehydration and enoylreduction take place after the fourth condensation step, as determined by the presence of the DH and ER domains in module 4. Once the polyketide chain is fully synthesized, it is released from the PKS through the action of the TE domain present at the end of module 6 and cyclizes to form the macrocyclic lactone 6-deoxyerythronolide B which is subsequently acted upon by a series of other enzymes, whose genes reside in the erythromycin cluster of the *Sac. erythraea* chromosome (see FIG. 1). As shown in FIG. 1, erythromycin carries methyl side chains at position 2, 4, 6, 8, 10 and 12, through the incorporation of methylmalonate as the extender unit at each step of synthesis of the polyketide moiety.

In the present invention, novel polyketide molecules of a desired structure are produced by introducing specific genetic alterations into a PKS-encoding sequence in the genome of a polyketide-producing microorganism. Alteration of one or more genes or fragments thereof may be generated through manipulation of genes residing exclusively within a species (i.e. intraspecies alterations), and include not only manipulations of genes within a single PKS cluster but also between different PKS clusters residing within a single strain (as is seen in *S. hygroscopicus*). Several examples of intraspecies alterations showing the manipulation of genes exclusively within a single PKS (namely, *eryA*) are described in U.S. application Serial No. 07/624,734 cited *supra*. Alternatively, a gene or fragment thereof may be exchanged with a heterologous gene or gene fragment encoding one or more related

domains from the PKS of a different polyketide-producing microorganism (interspecies alterations). Several examples of novel polyketides produced from exchange of heterologous genes are provided herein.

Whether the genetic manipulations are performed intraspecies or interspecies, three types of alterations to a PKS sequence may be carried out: (i) those which affect a module but do not cause the arrest of chain growth (Type I alterations); (ii) those which affect a single function in a module thereby causing the arrest of chain growth (Type II alterations); and (iii) those which affect an entire module (Type III alterations). In one embodiment, Type I alterations are produced by inactivation of domains that specify the functional groups and/or degree of oxidation found at specific ring positions in the native polyketide. Such domains typically include  $\beta$ -ketoreductases, dehydratases and enoylreductases. For example, an allele corresponding to  $\beta$ -ketoreductase of module 5 may be mutated by deleting a substantial portion of the DNA encoding the  $\beta$ -ketoreductase (thereby producing an inactive domain) and used to replace the wild-type allele in the native strain. Such a transfer results in the production of the novel polyketide 5-oxo-5,6-dideoxy-3- $\infty$ -mycarosyl erythronolide B.

In an alternative embodiment, Type I alterations are generated by replacing at least one domain in a particular PKS with at least one related domain from the same or a second PKS. Such related domains may exist between different polyketide-producing microorganisms (such as for example, the AT domains of *Sac. erythraea*, *S. venezuelae*, *S. hygroscopicus*, and *S. caelestis*) or within a single species (as for example, the LigAT2 and rapAT1 domains in *S. hygroscopicus*).

Ways to identify polyketide synthases, their domains and the functional similarity of domains are well-known to those of ordinary skill in the art. For example, the PKS region of the chromosome of a polyketide or putative polyketide-producing microorganism may be identified by hybridizing with nucleic acid probes under conditions of low or high stringency. Hybridization under high stringency conditions is generally performed in a buffer consisting of 15 mM sodium chloride and 1.5 mM trisodium citrate (0.1 x SSC) with an incubation temperature of about 65°C (see for example, Maniatis, *et al. supra*). To detect more distantly related PKS genes, hybridization is performed under low stringency conditions which include lower temperature incubations and/or the presence of increased amounts of sodium chloride and trisodium citrate (Maniatis, *et al. supra*). Once identified, the chromosomal region may be isolated, cloned into a suitable vector and sequenced, using conventional methods or commercial sequencing kits such as Sequenase (US Biochemical Corp, Cleveland, OH). Methods for isolating and cloning chromosomal DNA are also well known in the art (Maniatis, *et al. supra*). An amino acid sequence may then be deduced from the DNA sequence and a comparison made of the unknown amino acid sequence to that of one or more polypeptides involved in polyketide biosynthesis. Two amino acid sequences showing at least about 20% and more preferably about 25% identity and having conserved active site

residues or motifs are considered to specify functionally similar or equivalent PKS domains. Having identified such domains, the number and composition of modules as well as the arrangement of modules within particular ORFs can be determined.

In the case where the newly defined PKS produces a polyketide of known structure, the  $\beta$ -carbonyl processing and types of side chain moieties and their positioning on the polyketide backbone can be correlated to specific domains within modules. Because modules are established linearly within ORFs, this correlation also allows one to determine the order of modular activity (i.e. which module catalyzes which condensation step) in the PKS. For example, the  $\beta$ -carbonyl processing and types of side chain moieties in the polyketide generates a pattern of chemical groups that can be correlated to a pattern of domains within an ORF. Based on the specific type of side chain moiety at a given carbon, one can then predict the particular substrate utilized by that module's AT domain.

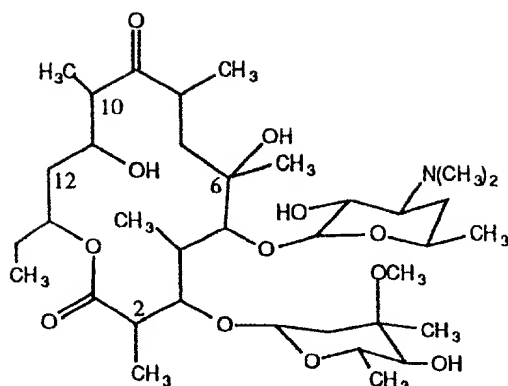
In the case where the polyketide structure is unknown, theoretically, comparative sequence analysis alone may be used to predict the substrate specificity of an AT domain. To accomplish this, at least two and preferably, three or more sequences known or predicted to specify a particular substrate can be compared to determine one or more conserved or consensus motifs unique to that family of ATs. An unknown AT having such motifs can then be assigned to a particular family.

Alternatively, comparative analyses can be performed using computer programs which group AT domains based on primary amino acid sequence similarity or phylogenetic relationships. For example, comparative analyses were made of the amino acid sequences of the AT domains in DEBS with corresponding AT domains in the PKS for rapamycin to determine whether the extender unit used by a particular AT domain, (either malonate or methylmalonate), correlated with the degree of sequence identity between these domains. Rapamycin is a large polyketide that is assembled through 14 condensation events; the rapamycin PKS possesses 14 AT domains whose sequences were deduced from known nucleotide sequences (Aparicio *et al.* *Gene* **169**:9-16 (1996)). Amino acid sequence comparisons of the 14 AT domains of the rapamycin PKS with each other and with the 6 AT domains from DEBS, showed that the AT domains fell into two distinct groupings in which the rapamycin AT domains from modules 1, 3, 4, 6, 7, 10 and 13 clustered with the 6 erythromycin AT domains and the rapamycin AT domains in modules 2, 5, 8, 9, 11, 12 and 14 formed a separate cluster (Haydock *et al.* *FEBS Letts.* **374**:246-248 (1995)). Examination of the polyketide structure of rapamycin indicated that methyl side chains were at positions on the lactone ring corresponding to condensation steps 1, 3, 4, 6, 7, 10 and 13, which suggested that methylmalonate was used as the extender unit during synthesis of these sections of the acyl chain; protons at the positions of the lactone ring corresponding to condensations steps 2, 5, 8, 9, 11, 12 and 14 suggested that malonate was utilized as the extender unit during synthesis of these sections. Two additional AT domains described

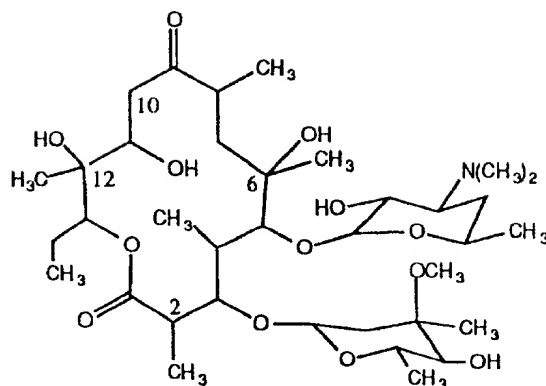
herein, ligAT2 and venAT, were also found to cluster with the putative malonate AT domains from the rapamycin PKS (FIG. 3). Having predicted that AT domains from rap modules 2, 5, 8, 9, 11, 12 or 14, as well as ligAT2 and venAT, specify malonate as extender units, the DNA encoding such domains could be isolated, cloned and used to replace the DNA encoding one or more AT domains in a PKS such as DEBS, in order to generate novel polyketides.

The techniques for determining the amino acid sequence "similarity" are well-known in the art. In general, when two or more polypeptides are aligned with one another, their sequence similarity refers to the amino acids at corresponding positions within each polypeptide sequence that are identical or possess similar chemical and/or physical properties such as charge or hydrophobicity. A so-termed "percent similarity" then can be determined between the compared polypeptide sequences. In general, the term "identity" refers to an exact nucleotide to nucleotide or amino acid to amino acid correspondence at a given position of two polynucleotides or polypeptide sequences, respectively. Two amino acid sequences (or for that matter, two or more polynucleotide sequences) can be compared by determining their "percent identity." The programs available in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group (GCG), Madison, WI), for example, the GAP program, are capable of calculating both the identity between two polynucleotides and the identity and similarity between two polypeptide sequences, respectively. Other programs for calculating and displaying similarity between sequences are known in the art. For example, the Growtree program (GCG, Madison, WI) creates a phylogenetic tree wherein the most closely related sequences are clustered and joined by the shortest lines. This tree is derived from a matrix created by the program Distances (GCG, Madison, WI) which calculates pairwise relationships within a group of aligned sequences.

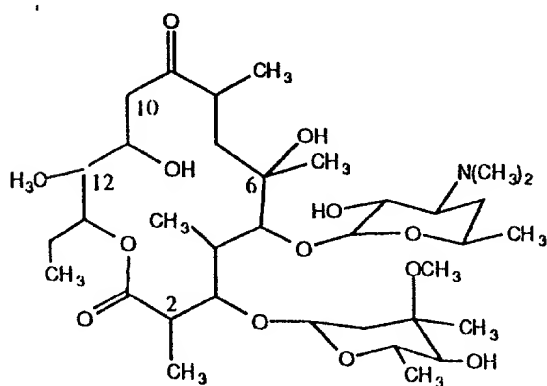
In a preferred embodiment, novel polyketide molecules of desired structure are produced by the replacement of at least one AT domain-encoding fragment of DNA of the *Sac. erythraea* chromosome with at least one heterologous AT domain-encoding fragment of DNA from another PKS cluster to yield novel polyketide compounds which are derivatives of 6-deoxyerythronolide B, erythronolide B, 3- $\alpha$ -L-mycarosylerythronolide B, or erythromycins A, B, C and D. Such derivatives are compounds wherein methyl (-Me) side chains at one or more positions of the macrocyclic lactone ring are replaced by substituents independently selected from the group consisting of (a) -H; (b) ethyl group (-Et); (c) hydroxyl group (-OH) and (d) allyl group (-Al). In a particularly preferred embodiment, a method is provided for the genetic modification of erythromycin-producing microorganisms which enables them to produce the novel compounds 12-desmethyl-12-deoxyerythromycin A, 10-desmethylerythromycin A, 10-desmethyl-12-deoxyerythromycin A, or 6-desmethyl-6-ethylerythromycin A. The compounds 12-desmethyl-12-deoxyerythromycin A, 10-desmethylerythromycin A, 10-desmethyl-12-deoxyerythromycin A, and 6-desmethyl-6-ethylerythromycin A are represented by the structural formulae:



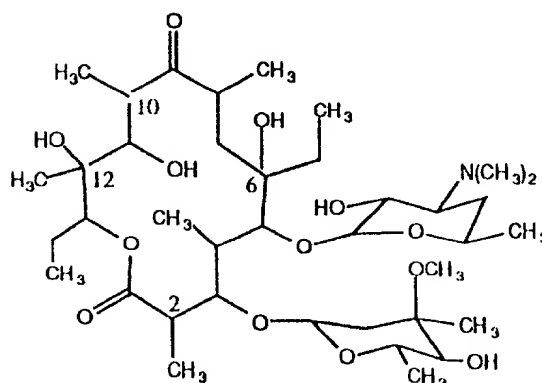
12-desmethyl-12-deoxyerythromycin A (I)



10-desmethylethylerythromycin A (II)



10-desmethyl-12-deoxyerythromycin A (III)



6-desmethyl-6-ethylerythromycin A (IV)

The general scheme for producing such polyketides is outlined in FIG. 4a and FIG. 4b. In the preferred embodiment, heterologous DNA fragments encoding related AT domains are introduced into the *Sac. erythraea* chromosome by a two-step method termed gene replacement.

In the first step of gene replacement, an integration vector is constructed through a multi-step cloning approach that places a heterologous gene or fragment thereof between two segments of DNA having sequences which are identical to those that immediately border (on each side) the resident polynucleotide sequence to be replaced. Construction of such a vector may be achieved by any means known to those of ordinary skill in art. For example, nucleotide sequences which flank the gene to be replaced can be generated by PCR amplification using chromosomal DNA as template and primers which hybridize to the chromosomal sequences immediately upstream and downstream of the flanking sequences of interest. The length of the flanking sequences is not critical to the practice of the invention but preferably is about 20-5000 base pairs (bp), more preferably about 100-5000 bp, and even more preferably about 500-5000 bp. A most preferred length of flanking sequence is about



750-1500 bp. Primers used for such amplifications may also comprise convenient restriction sites to facilitate cloning of the amplified sequences into suitable preparative vectors, to facilitate insertion of the heterologous sequence of interest between the flanking sequences and/or to facilitate subcloning of the entire group of sequences (5'-flanking region/heterologous polynucleotide sequence of interest/flanking region-3') into suitable vectors for integration. The desired heterologous polynucleotide sequences may be generated in a like manner.

The integration vectors are constructed to also comprise a fragment of DNA containing at least one origin of replication that is functional in an intermediate host but is non-functional or poorly functional in the production host. The vectors further comprise one or more fragments of DNA conferring resistance to an antibiotic, of which at least one functions in the intermediate host and at least one functions in the production host. Preferred integration vectors comprise the ColE1 and pIJ101 origins of replication, as found in plasmid pCS5 (J. Vara *et al.*, *J. Bacteriol.* **171**:5872-5881 (1989)). A particularly preferred vector carries a DNA fragment conferring resistance to thiostrepton and ampicillin. However, those skilled in the art understand that the particular antibiotic resistance genes and origins of replication identified above are necessary only inasmuch as they allow for the generation and selection of the desired recombinant plasmids and host cells. Other markers and origins of replication may also be used in the practice of the invention.

When the resident domains of a PKS are functional components of large multifunctional polypeptides, care must be taken in the construction of the integration plasmid so that the heterologous DNA fragment encoding the heterologous AT domain is positioned in the correct orientation and reading frame to its flanking DNA segments so that upon translation from the beginning of the coding sequence, an enzymatically functional protein is produced. The correct positioning becomes immediately apparent from knowledge of the nucleotide sequences of the host PKS genes and the heterologous genes used for gene replacement.

In the second step, each of the integration vectors carrying a related gene or fragment thereof is independently introduced into a host strain and recombination between each of the genomic fragments in the integration plasmid and its corresponding homologous fragment in the host strain chromosome is allowed to occur. This procedure results in the exchange of the resident AT-encoding DNA in the chromosome for its heterologous counterpart. The general scheme for gene replacement by homologous recombination is outlined in FIG. 5.

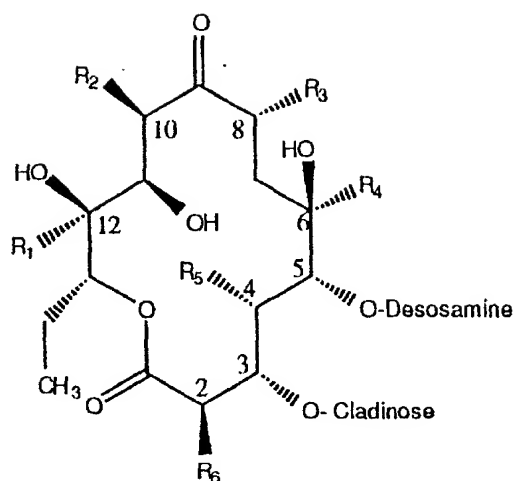
Procedures to introduce DNA into polyketide-producing microorganisms and to facilitate homologous recombination are described herein. However, those skilled in the art understand that alternative procedures for introducing DNA into a polyketide-producing microorganism, such as electroporation, transduction, or conjugation, are well known and may also be used in the practice of the invention. Procedures for cultivating polyketide-

producing microorganisms, as well as methods to recover novel polyketides produced from modified strains, to purify such compounds and to confirm the identity of those compounds (such as by mass spectrometry or NMR) are well-known to those of ordinary skill in the art.

Although the present invention is described in the Examples that follow in terms of preferred embodiments, they are not to be regarded as limiting the scope of the invention. The descriptions that follow serve to illustrate the principles and methodologies involved in creating novel derivatives of erythromycin. Whereas the examples below describe the replacement of the *Sac. erythraea* AT1, AT2, and AT4-encoding DNA fragments with a heterologous DNA fragment which encodes either an AT domain that specifies incorporation of malonate (malonate-AT) or an AT domain that specifies incorporation of ethylmalonate (ethylmalonate-AT), those skilled in the art understand that one or more fragments of heterologous DNA encoding malonate, ethylmalonate, allylmalonate, and/or hydroxymalonnate (tartronate)-AT domains can be used to replace the other AT-encoding DNA fragments of the erythromycin PKS in *Sac. erythraea* to result in the production of other novel erythromycin derivatives. For example, novel erythromycins produced when resident AT-encoding DNA fragments in the erythromycin PKS (eryPKS) are independently replaced with heterologous DNA fragments specifying malonate and/or ethylmalonnate as the extender unit are shown in Table 1.

In particular, those skilled in the art understand that following the methods described herein for replacement of a single resident AT-encoding DNA fragment in the eryPKS, replacements of two resident AT-encoding DNA fragments with heterologous DNA fragments (specifying malonnate, ethylmalonnate, allylmalonnate, and/or hydroxymalonnate -AT domains) in stepwise fashion are also possible and result in the formation of novel disubstituted erythromycins. Similarly, trisubstituted erythromycins, tetrasubstituted erythromycins, pentasubstituted erythromycins and hexasubstituted erythromycins can also be made by replacement of three, four, five and six resident AT-encoding DNA fragments in the eryPKS, respectively, with heterologous AT-encoding DNA fragments as described herein. Therefore, all substitutions of AT-encoding DNA fragments in the eryPKS with heterologous AT-encoding DNA fragments (yielding all varieties of proton, ethyl, allyl, and hydroxyl substituted erythromycin derivatives) are within the scope of the present invention. Examples of compounds produced by such replacements include but are not limited to those shown in Table 1 below.

Table 1  
Structures from Changes at Side Chain Positions



## A. Single Changes

	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	R <sub>6</sub>	Name
5	H	Me	Me	Me	Me	Me	12-Desmethylethylerythromycin A
	Et	Me	Me	Me	Me	Me	12-Desmethyl-12-ethylerythromycin A
	Me	H	Me	Me	Me	Me	10-Desmethylethylerythromycin A
	Me	Et	Me	Me	Me	Me	10-Desmethyl-10-ethylerythromycin A
10	Me	Me	H	Me	Me	Me	8-Desmethylethylerythromycin A
	Me	Me	Et	Me	Me	Me	8-Desmethyl-8-ethylerythromycin A
	Me	Me	Me	H	Me	Me	6-Desmethylethylerythromycin A
	Me	Me	Me	Et	Me	Me	6-Desmethyl-6-ethylerythromycin A
15	Me	Me	Me	Me	H	Me	4-Desmethylethylerythromycin A
	Me	Me	Me	Me	Et	Me	4-Desmethyl-4-ethylerythromycin A
	Me	Me	Me	Me	Me	H	2-Desmethylethylerythromycin A
	Me	Me	Me	Me	Me	Et	2-Desmethyl-2-ethylerythromycin A

## B. Two Changes

20	H	Me	Me	Me	Me	Et	2,12-Didesmethyl-2-ethylerythromycin A
	H	Me	Me	Me	Et	Me	4,12-Didesmethyl-4-ethylerythromycin A
	H	Me	Me	Et	Me	Me	6,12-Didesmethyl-6-ethylerythromycin A
	H	Me	Et	Me	Me	Me	8,12-Didesmethyl-8-ethylerythromycin A
25	H	Et	Me	Me	ME	Me	10,12-Didesmethyl-10-ethylerythromycin A
	H	Me	Me	Me	Me	H	2,12-Didesmethylethylerythromycin A
	H	Me	Me	Me	H	Me	4,12-Didesmethylethylerythromycin A
	H	Me	Me	H	Me	Me	6,12-Didesmethylethylerythromycin A
30	H	Me	H	Me	Me	Me	8,12-Didesmethylethylerythromycin A
	H	H	Me	Me	Me	Me	10,12-Didesmethylethylerythromycin A
	Me	H	Me	Me	Me	Et	2,10-Didesmethyl-2-ethylerythromycin A
	Me	H	Me	Me	Et	Me	4,10-Didesmethyl-4-ethylerythromycin A
35	Me	H	Me	Et	Me	Me	6,10-Didesmethyl-6-ethylerythromycin A
	Me	H	Et	Me	Me	Me	8,10-Didesmethyl-8-ethylerythromycin A
	Me	H	Me	Me	Me	H	2,10-Didesmethylethylerythromycin A
	Me	H	Me	Me	H	Me	4,10-Didesmethylethylerythromycin A
40	Me	H	Me	H	Me	Me	6,10-Didesmethylethylerythromycin A
	Me	H	H	Me	Me	Me	8,10-Didesmethylethylerythromycin A
	Me	Me	H	Me	Me	Et	2,8-Didesmethyl-2-ethylerythromycin A
	Me	Me	H	Me	Et	Me	4,8-Didesmethyl-4-ethylerythromycin A
40	Me	Me	H	Et	Me	Me	6,8-Didesmethyl-6-ethylerythromycin A
	Me	Me	H	Me	Me	H	2,8-Didesmethylethylerythromycin A
	Me	Me	H	Me	H	Me	4,8-Didesmethylethylerythromycin A
	Me	Me	H	H	Me	Me	6,8-Didesmethylethylerythromycin A

5	Me	Me	Me	H	Me	Et	2,6-Didesmethyl-2-ethylerythromycin A
	Me	Me	Me	H	Et	Me	4,6-Didesmethyl-4-ethylerythromycin A
	Me	Me	Me	H	Me	H	2,6-Didesmethylethylerythromycin A
	Me	Me	Me	H	H	Me	4,6-Didesmethylethylerythromycin A
	Me	Me	Me	Me	H	Et	2,4,-Didesmethyl-2-ethylerythromycin A
10	Me	Me	Me	Me	H	H	2,4,-Didesmethylethylerythromycin A
	Et	Me	Me	Me	Me	Et	2,12-Didesmethyl-2,12-diethylerythromycin A
	Et	Me	Me	Me	Et	Me	4,12-Didesmethyl-4,12-diethylerythromycin A
	Et	Me	Me	Et	Me	Me	6,12-Didesmethyl-6,12-diethylerythromycin A
	Et	Me	Et	Me	Me	Me	8,12-Didesmethyl-8,12-diethylerythromycin A
15	Et	Et	Me	Me	Me	Me	10,12-Didesmethyl-10,12-diethylerythromycin A
	Et	Me	Me	Me	Me	H	2,12-Didesmethyl-12-ethylerythromycin A
	Et	Me	Me	Me	H	Me	4,12-Didesmethyl-12-ethylerythromycin A
	Et	Me	Me	H	Me	Me	6,12-Didesmethyl-12-ethylerythromycin A
	Et	Me	H	Me	Me	Me	8,12-Didesmethyl-12-ethylerythromycin A
20	Et	H	Me	Me	Me	Me	10,12-Didesmethyl-12-ethylerythromycin A
	Me	Et	Me	Me	Me	Et	2,10-Didesmethyl-2,10-diethylerythromycin A
	Me	Et	Me	Me	Et	Me	4,10-Didesmethyl-4,10-diethylerythromycin A
	Me	Et	Me	Et	Me	Me	6,10-Didesmethyl-6,10-diethylerythromycin A
	Me	Et	Et	Me	Me	Me	8,10-Didesmethyl-8,10-diethylerythromycin A
25	Me	Et	Me	Me	Me	H	2,10-Didesmethyl-10-ethylerythromycin A
	Me	Et	Me	Me	H	Me	4,10-Didesmethyl-10-ethylerythromycin A
	Me	Et	Me	H	Me	Me	6,10-Didesmethyl-10-ethylerythromycin A
	Me	Et	H	Me	Me	Me	8,10-Didesmethyl-10-ethylerythromycin A
	Me	Me	Et	Me	Me	Et	2,8-Didesmethyl-2,8-diethylerythromycin A
30	Me	Me	Et	Me	Et	Me	4,8-Didesmethyl-4,8-diethylerythromycin A
	Me	Me	Et	Et	Me	Me	6,8-Didesmethyl-6,8-diethylerythromycin A
	Me	Me	Et	Me	Me	H	2,8-Didesmethyl-8-ethylerythromycin A
	Me	Me	Et	Me	H	Me	4,8-Didesmethyl-8-ethylerythromycin
	Me	Me	Et	H	Me	Me	6,8-Didesmethyl-8-ethylerythromycin
35	Me	Me	Me	Et	Me	Et	2,6-Didesmethyl-2,6-diethylerythromycin A
	Me	Me	Me	Et	Et	Me	4,6-Didesmethyl-4,6-diethylerythromycin A
	Me	Me	Me	Et	Me	H	2,6-Didesmethyl-6-ethylerythromycin A
	Me	Me	Me	Et	H	Me	4,6-Didesmethyl-6-ethylerythromycin
	Me	Me	Me	Me	Et	Et	2,4-Didesmethyl-2,4-diethylerythromycin A
	Me	Me	Me	Me	Et	H	2,4-Didesmethyl-4-ethylerythromycin A

## C. Three Changes

40	H	H	Me	Me	Me	Et	2,10,12-Tridesmethyl-2-Ethylerythromycin A
	H	H	Me	Me	Me	H	2,10,12-Tridesmethylethylerythromycin A
	H	H	Me	Me	Et	Me	4,10,12-Tridesmethyl-4-Ethylerythromycin A
	H	H	Me	Me	H	Me	4,10,12-Tridesmethylethylerythromycin A
	H	H	Me	Et	Me	Me	6,10,12-Tridesmethyl-6-Ethylerythromycin A
45	H	H	Me	H	Me	Me	6,10,12-Tridesmethylethylerythromycin A
	H	H	Et	Me	Me	Me	8,10,12-Tridesmethyl-8-ethylerythromycin A
	H	H	H	Me	Me	Me	8,10,12-Tridesmethylethylerythromycin A
	Et	H	Me	Me	Me	Et	2,10,12-Tridesmethyl-2,12,-diethylerythromycin A
	Et	H	Me	Me	Me	H	2,10,12-Tridesmethyl-12-ethylerythromycin A
50	Et	H	Me	Me	Et	Me	4,10,12-Tridesmethyl-4,12-diethylerythromycin A
	Et	H	Me	Me	H	Me	4,10,12-Tridesmethyl-12-ethylerythromycin A
	Et	H	Me	Et	Me	Me	6,10,12-Tridesmethyl-6,12-diethylerythromycin A
	Et	H	Me	H	Me	Me	6,10,12-Tridesmethyl-12-ethylerythromycin A
	Et	H	Et	Me	Me	Me	8,10,12-Tridesmethyl-8,12-diethylerythromycin A
55	Et	H	H	Me	Me	Me	8,10,12-Tridesmethyl-12-ethylerythromycin A
	H	Et	Me	Me	Me	Et	2,10,12-Tridesmethyl-2,10-diethylerythromycin A
	H	Et	Me	Me	Me	H	2,10,12-Tridesmethyl-10-ethylerythromycin A

5	H	Et	Me	Me	Et	Me	4,10,12-Tridesmethyl-4,10-diethylerythromycin A
	H	Et	Me	Me	H	Me	4,10,12-Tridesmethyl-10-ethylerythromycin A
	H	Et	Me	Et	Me	Me	6,10,12-Tridesmethyl-6,10-diethylerythromycin A
	H	Et	Me	H	Me	Me	6,10,12-Tridesmethyl-10-ethylerythromycin A
	H	Et	Et	Me	Me	Me	8,10,12-Tridesmethyl-8,10-diethylerythromycin A
10	H	Et	H	Me	Me	Me	8,10,12-Tridesmethyl-10-ethylerythromycin A
	Et	Et	Me	Me	Me	Et	2,10,12-Tridesmethyl-2,10,12-triethylerythromycin A
	Et	Et	Me	Me	Me	H	2,10,12-Tridesmethyl-10,12-diethylerythromycin A
	Et	Et	Me	Me	Et	Me	4,10,12-Tridesmethyl-4,10,12-triethylerythromycin A
	Et	Et	Me	Me	H	Me	4,10,12-Tridesmethyl-10,12-diethylerythromycin A
15	Et	Et	Me	Et	Me	Me	6,10,12-Tridesmethyl-6,10,12-triethylerythromycin A
	Et	Et	Me	H	Me	Me	6,10,12-Tridesmethyl-10,12-diethylerythromycin A
	Et	Et	Et	Me	Me	Me	8,10,12-Tridesmethyl-8,10,12-triethylerythromycin A
	Et	Et	H	Me	Me	Me	8,10,12-Tridesmethyl-10,12-diethylerythromycin A
	H	Me	H	Me	Me	Et	2,8,12-Tridesmethyl-2-ethylerythromycin A
20	H	Me	H	Me	Me	H	2,8,12-Tridesmethylethylerythromycin A
	H	Me	H	Me	Et	Me	4,8,12-Tridesmethyl-4-ethylerythromycin A
	H	Me	H	Me	H	Me	4,8,12-Tridesmethylethylerythromycin A
	H	Me	H	Et	Me	Me	6,8,12-Tridesmethyl-6-ethylerythromycin A
	H	Me	H	H	Me	Me	6,8,12-Tridesmethylethylerythromycin A
25	Et	Me	H	Me	Me	Et	2,8,12-Tridesmethyl-2,12-diethylerythromycin A
	Et	Me	H	Me	Me	H	2,8,12-Tridesmethyl-12-ethylerythromycin A
	Et	Me	H	Me	Et	Me	4,8,12-Tridesmethyl-4,12-diethylerythromycin A
	Et	Me	H	Me	H	Me	4,8,12-Tridesmethyl-12-ethylerythromycin A
	Et	Me	H	Et	Me	Me	6,8,12-Tridesmethyl-6,12-diethylerythromycin A
30	Et	Me	H	H	Me	Me	6,8,12-Tridesmethyl-12-ethylerythromycin A
	H	Me	Et	Me	Me	Et	2,8,12-Tridesmethyl-2,8-diethylerythromycin A
	H	Me	Et	Me	Me	H	2,8,12-Tridesmethyl-8-ethylerythromycin A
	H	Me	Et	Me	Et	Me	4,8,12-Tridesmethyl-4,8-diethylerythromycin A
	H	Me	Et	Me	H	Me	4,8,12-Tridesmethyl-8-ethylerythromycin A
35	H	Me	Et	Et	Me	Me	6,8,12-Tridesmethyl-6,8-diethylerythromycin A
	H	Me	Et	H	Me	Me	6,8,12-Tridesmethyl-8-ethylerythromycin A
	Et	Me	Et	Me	Me	Et	2,8,12-Tridesmethyl-2,8,12-triethylerythromycin A
	Et	Me	Et	Me	Me	H	2,8,12-Tridesmethyl-8,12-diethylerythromycin A
	Et	Me	Et	Me	Et	Me	4,8,12-Tridesmethyl-4,8,12-triethylerythromycin A
40	Et	Me	Et	Me	H	Me	4,8,12-Tridesmethyl-8,12-diethylerythromycin A
	Et	Me	Et	Et	Me	Me	6,8,12-Tridesmethyl-6,8,12-triethylerythromycin A
	Et	Me	Et	H	Me	Me	6,8,12-Tridesmethyl-8,12-diethylerythromycin A
	H	Me	Me	H	Me	Et	2,6,12-Tridesmethyl-2-ethylerythromycin A
	H	Me	Me	H	Me	H	2,6,12-Tridesmethylethylerythromycin A
45	H	Me	Me	H	Et	Me	4,6,12-Tridesmethyl-4-ethylerythromycin A
	H	Me	Me	H	H	Me	4,6,12-Tridesmethylethylerythromycin A
	Et	Me	Me	H	Me	Et	2,6,12-Tridesmethyl-2,12-diethylerythromycin A
	Et	Me	Me	H	Me	H	2,6,12-Tridesmethyl-12-ethylerythromycin A
	Et	Me	Me	H	Et	Me	4,6,12-Tridesmethyl-4,12-diethylerythromycin A
50	Et	Me	Me	H	H	Me	4,6,12-Tridesmethyl-12-ethylerythromycin A
	H	Me	Me	Et	Me	Et	2,6,12-Tridesmethyl-2,6-diethylerythromycin A
	H	Me	Me	Et	Me	H	2,6,12-Tridesmethyl-6-ethylerythromycin A
	H	Me	Me	Et	Et	Me	4,6,12-Tridesmethyl-4,6-diethylerythromycin A
	H	Me	Me	Et	H	Me	4,6,12-Tridesmethyl-6-ethylerythromycin A
55	Et	Me	Me	Et	Me	Et	2,6,12-Tridesmethyl-2,6,12-triethylerythromycin A
	Et	Me	Me	Et	Me	H	2,6,12-Tridesmethyl-6,12-diethylerythromycin A
	Et	Me	Me	Et	Et	Me	4,6,12-Tridesmethyl-4,6,12-triethylerythromycin A
	Et	Me	Me	Et	H	Me	4,6,12-Tridesmethyl-6,12-diethylerythromycin A
	H	Me	Me	Me	H	Et	2,4,12-Tridesmethyl-2-ethylerythromycin A
	H	Me	Me	Me	H	H	2,4,12-Tridesmethylethylerythromycin A

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	Et	Me	Me	Me	H	Et	2,4,12-Tridesmethyl-2,12-diethylerythromycin A
	Et	Me	Me	Me	H	H	2,4,12-Tridesmethyl-12-ethylerythromycin A
	H	Me	Me	Me	Et	Et	2,4,12-Tridesmethyl-2,4-diethylerythromycin A
	H	Me	Me	Me	Et	H	2,4,12-Tridesmethyl-4-ethylerythromycin A
5	Et	Me	Me	Me	Et	Et	2,4,12-Tridesmethyl-2,4,12-triethylerythromycin A
	Et	Me	Me	Me	Et	H	2,4,12-Tridesmethyl-2,12-diethylerythromycin A
	Me	H	H	Me	Me	Et	2,8,10-Tridesmethyl-2-ethylerythromycin A
	Me	H	H	Me	Me	H	2,8,10-Tridesmethylethylerythromycin A
	Me	H	H	Me	Et	Me	4,8,10-Tridesmethyl-4-ethylerythromycin A
10	Me	H	H	Me	H	Me	4,8,10-Tridesmethylethylerythromycin A
	Me	H	H	Et	Me	Me	6,8,10-Tridesmethyl-6-ethylerythromycin A
	Me	H	H	H	Me	Me	6,8,10-Tridesmethylethylerythromycin A
	Me	Et	H	Me	Me	Et	2,8,10-Tridesmethyl-2,10-diethylerythromycin A
	Me	Et	H	Me	Me	H	2,8,10-Tridesmethyl-10-ethylerythromycin A
15	Me	Et	H	Me	Et	Me	4,8,10-Tridesmethyl-4,10-diethylerythromycin A
	Me	Et	H	Me	H	Me	4,8,10-Tridesmethyl-10-ethylerythromycin A
	Me	Et	H	Et	Me	Me	6,8,10-Tridesmethyl-6,10-diethylerythromycin A
	Me	Et	H	H	Me	Me	6,8,10-Tridesmethyl-10-ethylerythromycin A
	Me	H	Et	Me	Me	Et	2,8,10-Tridesmethyl-2,8-diethylerythromycin A
20	Me	H	Et	Me	Me	H	2,8,10-Tridesmethyl-8-ethylerythromycin A
	Me	H	Et	Me	Et	Me	4,8,10-Tridesmethyl-4,8-diethylerythromycin A
	Me	H	Et	Me	H	Me	4,8,10-Tridesmethyl-8-ethylerythromycin A
	Me	H	Et	Et	Me	Me	6,8,10-Tridesmethyl-6,8-diethylerythromycin A
	Me	H	Et	H	Me	Me	6,8,10-Tridesmethyl-8-ethylerythromycin A
25	Me	Et	Et	Me	Me	Et	2,8,10-Tridesmethyl-2,8,10-triethylerythromycin A
	Me	Et	Et	Me	Me	H	2,8,10-Tridesmethyl-8,10-diethylerythromycin A
	Me	Et	Et	Me	Et	Me	4,8,10-Tridesmethyl-4,8,10-triethylerythromycin A
	Me	Et	Et	Me	H	Me	4,8,10-Tridesmethyl-8-10-diethylerythromycin A
	Me	Et	Et	Et	Me	Me	6,8,10-Tridesmethyl-6,8,10-triethylerythromycin A
30	Me	Et	Et	H	Me	Me	6,8,10-Tridesmethyl-8,10-diethylerythromycin A
	Me	H	Me	H	Me	Et	2,6,10-Tridesmethyl-2-ethylerythromycin A
	Me	H	Me	H	Me	H	2,6,10-Tridesmethylethylerythromycin A
	Me	H	Me	H	Et	Me	4,6,10-Tridesmethyl-4-ethylerythromycin A
	Me	H	Me	H	H	Me	4,6,10-Tridesmethylethylerythromycin A
35	Me	Et	Me	H	Me	Et	2,6,10-Tridesmethyl-2,1--diethylerythromycin A
	Me	Et	Me	H	Me	H	2,6,10-Tridesmethyl-10-ethylerythromycin A
	Me	Et	Me	H	Et	Me	4,6,10-Tridesmethyl-4,10-diethylerythromycin A
	Me	Et	Me	H	H	Me	4,6,10-Tridesmethyl-10-ethylerythromycin A
	Me	H	Me	Et	Me	Et	2,6,10-Tridesmethyl-2,6-diethylerythromycin A
40	Me	H	Me	Et	Me	H	2,6,10-Tridesmethyl-6-ethylerythromycin A
	Me	H	Me	Et	Et	Me	4,6,10-Tridesmethyl-4,6,diethylerythromycin A
	Me	H	Me	Et	H	Me	4,6,10-Tridesmethyl-6-ethylerythromycin A
	Me	Et	Me	Et	Me	Et	2,6,10-Tridesmethyl-2,6,10-triethylerythromycin A
	Me	Et	Me	Et	Me	H	2,6,10-Tridesmethyl-6,10-diethylerythromycin A
45	Me	Et	Me	Et	Et	Me	4,6,10-Tridesmethyl-4,6,10-triethylerythromycin A
	Me	Et	Me	Et	H	Me	4,6,10-Tridesmethyl-6,10-diethylerythromycin A
	Me	H	Me	Me	H	Et	2,4,10-Tridesmethyl-2-ethylerythromycin A
	Me	H	Me	Me	H	H	2,4,10-Tridesmethylethylerythromycin A
	Me	Et	Me	Me	H	Et	2,4,10-Tridesmethyl-2,10-diethylerythromycin A
50	Me	Et	Me	Me	H	H	2,4,10-Tridesmethyl-10-ethylerythromycin A
	Me	H	Me	Me	Et	Et	2,4,10-Tridesmethyl-2,4-diethylerythromycin A
	Me	H	Me	Me	Et	H	2,4,10-Tridesmethyl-4-ethylerythromycin A
	Me	Et	Me	Me	Et	Et	2,4,10-Tridesmethyl-2,4,10-triethylerythromycin A
	Me	Et	Me	Me	Et	H	2,4,10-Tridesmethyl-4,10-diethylerythromycin A
55	Me	Me	H	H	Me	Et	2,6,8-Tridesmethyl-2-ethylerythromycin A
	Me	Me	H	H	Me	H	2,6,8-Tridesmethylethylerythromycin A

5	Me	Me	H	H	Et	Me	4,6,8-Tridesmethyl-4-ethylerythromycin A
	Me	Me	H	H	H	Me	4,6,8-Tridesmethylethylerythromycin A
	Me	Me	Et	H	Me	Et	2,6,8-Tridesmethyl-2,8-diethylerythromycin A
	Me	Me	Et	H	Me	H	2,6,8-Tridesmethyl-8-ethylerythromycin A
	Me	Me	Et	H	Et	Me	4,6,8-Tridesmethyl-4,8-diethylerythromycin A
10	Me	Me	Et	H	H	Me	4,6,8-Tridesmethyl-8-ethylerythromycin A
	Me	Me	H	Et	Me	Et	2,6,8-Tridesmethyl-2,6-diethylerythromycin A
	Me	Me	H	Et	Me	H	2,6,8-Tridesmethyl-6-ethylerythromycin A
	Me	Me	H	Et	Et	Me	4,6,8-Tridesmethyl-4,6-diethylerythromycin A
	Me	Me	H	Et	H	Me	4,6,8-Tridesmethyl-6-ethylerythromycin A
15	Me	Me	Et	Et	Me	Et	2,6,8-Tridesmethyl-2,6,8-triethylerythromycin A
	Me	Me	Et	Et	Me	H	2,6,8-Tridesmethyl-6,8-diethylerythromycin A
	Me	Me	Et	Et	Et	Me	4,6,8-Tridesmethyl-4,6,8-triethylerythromycin A
	Me	Me	Et	Et	H	Me	4,6,8-Tridesmethyl-6,8-triethylerythromycin A
	Me	Me	H	Me	H	Et	2,4,8-Tridesmethyl-2-ethylerythromycin A
20	Me	Me	H	Me	H	H	2,4,8-Tridesmethylethylerythromycin A
	Me	Me	Et	Me	H	Et	2,4,8-Tridesmethyl-2,8-diethylerythromycin A
	Me	Me	Et	Me	H	H	2,4,8-Tridesmethyl-8-ethylerythromycin A
	Me	Me	H	Me	Et	Et	2,4,8-Tridesmethyl-2,4-diethylerythromycin A
	Me	Me	H	Me	Et	H	2,4,8-Tridesmethyl-4-ethylerythromycin A
25	Me	Me	Et	Me	Et	Et	2,4,8-Tridesmethyl-2,4,8-triethylerythromycin A
	Me	Me	Et	Me	Et	H	2,4,8-Tridesmethyl-4,8-diethylerythromycin A
	Me	Me	Me	H	H	Et	2,4,6-Tridesmethyl-2-ethylerythromycin A
	Me	Me	Me	H	H	H	2,4,6-Tridesmethylethylerythromycin A
	Me	Me	Me	Et	H	Et	2,4,6-Tridesmethyl-2,6-diethylerythromycin A
30	Me	Me	Me	Et	H	H	2,4,6-Tridesmethyl-6-ethylerythromycin A
	Me	Me	Me	H	Et	Et	2,4,6-Tridesmethyl-2,4-diethylerythromycin A
	Me	Me	Me	H	Et	H	2,4,6-Tridesmethyl-4-ethylerythromycin A
	Me	Me	Me	Et	Et	Et	2,4,6-Tridesmethyl-2,4,6-triethylerythromycin A
	Me	Me	Me	Et	Et	H	2,4,6-Tridesmethyl-4,6-diethylerythromycin A

## D. Four Changes

35	H	H	H	Me	Me	Et	2,8,10,12-Tetradesmethyl-2-ethylerythromycin A
	H	H	H	Me	Me	H	2,8,10,12-Tetradesmethylethylerythromycin A
	H	H	H	Me	Et	Me	4,8,10,12-Tetradesmethyl-4-ethylerythromycin A
	H	H	H	Me	H	Me	4,8,10,12-Tetradesmethylethylerythromycin A
	H	H	H	Et	Me	Me	6,8,10,12-Tetradesmethyl-6-ethylerythromycin A
40	H	H	H	H	Me	Me	6,8,10,12-Tetradesmethylethylerythromycin A
	H	Et	H	Me	Me	Et	2,6,10,12-Tetradesmethyl-2,10-diethylerythromycin A
	H	Et	H	Me	Me	H	2,6,10,12-Tetradesmethyl-10-ethylerythromycin A
	H	Et	H	Me	Et	Me	4,8,10,12-Tetradesmethyl-4,10-diethylerythromycin A
	H	Et	H	Me	H	Me	4,8,10,12-Tetradesmethyl-10-ethylerythromycin A
45	H	Et	H	Et	Me	Me	6,8,10,12-Tetradesmethyl-6,10-diethylerythromycin A
	H	Et	H	H	Me	Me	6,8,10,12-Tetradesmethyl-10-ethylerythromycin A
	H	H	Et	Me	Me	Et	2,8,10,12-Tetradesmethyl-2,8-diethylerythromycin A
	H	H	Et	Me	Me	H	2,8,10,12-Tetradesmethyl-8-ethylerythromycin A
	H	H	Et	Me	Et	Me	4,8,10,12-Tetradesmethyl-4,8-diethylerythromycin A
50	H	H	Et	Me	H	Me	4,8,10,12-Tetradesmethyl-8-ethylerythromycin A
	H	H	Et	Et	Me	Me	6,8,10,12-Tetradesmethyl-6,8-diethylerythromycin A
	H	H	Et	H	Me	Me	6,8,10,12-Tetradesmethyl-8-ethylerythromycin A
	H	Et	Et	Me	Me	Et	2,6,10,12-Tetradesmethyl-2,8,10-triethylerythromycin A
	H	Et	Et	Me	Me	H	2,6,10,12-Tetradesmethyl-8,10-diethylerythromycin A
55	H	Et	Et	Me	Et	Me	4,8,10,12-Tetradesmethyl-4,8,10-triethylerythromycin A
	H	Et	Et	Me	H	Me	4,8,10,12-Tetradesmethyl-8,10-diethylerythromycin A
	H	Et	Et	Et	Me	Me	6,8,10,12-Tetradesmethyl-6,8,10-triethylerythromycin A
	H	Et	Et	H	Me	Me	6,8,10,12-Tetradesmethyl-8,10-diethylerythromycin A

5	Et	H	H	Me	Me	Et	2,8,10,12-Tetradesmethyl-2,12-diethylerythromycin A
	Et	H	H	Me	Me	H	2,8,10,12-Tetradesmethyl-12-ethylerythromycin A
	Et	H	H	Me	Et	Me	4,8,10,12-Tetradesmethyl-4,12-diethylerythromycin A
	Et	H	H	Me	H	Me	4,8,10,12-Tetradesmethyl-12-ethylerythromycin A
	Et	H	H	Et	Me	Me	6,8,10,12-Tetradesmethyl-6,12-diethylerythromycin A
	Et	H	H	H	Me	Me	6,8,10,12-Tetradesmethyl-12-ethylerythromycin A
10	Et	Et	H	Me	Me	Et	2,6,10,12-Tetradesmethyl-2,10,12-triethylerythromycin A
	Et	Et	H	Me	Me	H	2,6,10,12-Tetradesmethyl-10,12-diethylerythromycin A
	Et	Et	H	Me	Et	Me	4,8,10,12-Tetradesmethyl-4,10,12-triethylerythromycin A
	Et	Et	H	Me	H	Me	4,8,10,12-Tetradesmethyl-10,12-diethylerythromycin A
	Et	Et	H	Et	Me	Me	6,8,10,12-Tetradesmethyl-6,10,12-triethylerythromycin A
	Et	Et	H	H	Me	Me	6,8,10,12-Tetradesmethyl-10,12-diethylerythromycin A
15	Et	H	Et	Me	Me	Et	2,8,10,12-Tetradesmethyl-2,8,12-triethylerythromycin A
	Et	H	Et	Me	Me	H	2,8,10,12-Tetradesmethyl-8,12-diethylerythromycin A
	Et	H	Et	Me	Et	Me	4,8,10,12-Tetradesmethyl-4,8,12-triethylerythromycin A
	Et	H	Et	Me	H	Me	4,8,10,12-Tetradesmethyl-8,12-diethylerythromycin A
	Et	H	Et	Et	Me	Me	6,8,10,12-Tetradesmethyl-6,8,12-triethylerythromycin A
	Et	H	Et	H	Me	Me	6,8,10,12-Tetradesmethyl-8,12-diethylerythromycin A
20	Et	Et	Et	Me	Me	Et	2,6,10,12-Tetradesmethyl-2,8,10,12-tetraethylerythromycin A
	Et	Et	Et	Me	Me	H	2,6,10,12-Tetradesmethyl-8,10,12-triethylerythromycin A
	Et	Et	Et	Me	Et	Me	4,8,10,12-Tetradesmethyl-4,8,10,12-tetraethylerythromycin A
	Et	Et	Et	Me	H	Me	4,8,10,12-Tetradesmethyl-8,10,12-triethylerythromycin
	Et	Et	Et	Et	Me	Me	6,8,10,12-Tetradesmethyl-6,8,10,12-tetraethylerythromycin A
	Et	Et	Et	H	Me	Me	6,8,10,12-Tetradesmethyl-8,10,12-triethylerythromycin
25	H	H	Me	H	Me	Et	2,6,10,12-Tetradesmethyl-2-ethylerythromycin A
	H	H	Me	H	Me	H	2,6,10,12-Tetradesmethylerythromycin A
	H	H	Me	H	Et	Me	4,6,10,12-Tetradesmethyl-4-ethylerythromycin A
	H	H	Me	H	H	Me	4,6,10,12-Tetradesmethylerythromycin A
	H	Et	Me	H	Me	Et	2,6,10,12-Tetradesmethyl-2,10-diethylerythromycin A
	H	Et	Me	H	Me	H	2,6,10,12-Tetradesmethyl-10-ethylerythromycin A
30	H	Et	Me	H	Et	Me	4,6,10,12-Tetradesmethyl-4,10-diethylerythromycin A
	H	Et	Me	H	H	Me	4,6,10,12-Tetradesmethyl-10-ethylerythromycin A
	H	H	Me	Et	Me	Et	2,6,10,12-Tetradesmethyl-2,6-diethylerythromycin A
	H	H	Me	Et	Me	H	2,6,10,12-Tetradesmethyl-6-ethylerythromycin A
	H	H	Me	Et	Et	Me	4,6,10,12-Tetradesmethyl-4,6-diethylerythromycin A
	H	H	Me	Et	H	Me	4,6,10,12-Tetradesmethyl-6-ethylerythromycin A
35	H	Et	Me	Et	Me	Et	2,6,10,12-Tetradesmethyl-2,6,10-triethylerythromycin A
	H	Et	Me	Et	Me	H	2,6,10,12-Tetradesmethyl-6,10-diethylerythromycin A
	H	Et	Me	Et	Et	Me	4,6,10,12-Tetradesmethyl-4,6,10-triethylerythromycin A
	H	Et	Me	Et	H	Me	4,6,10,12-Tetradesmethyl-6,10-diethylerythromycin A
	Et	H	Me	H	Me	Et	2,6,10,12-Tetradesmethyl-2,12-diethylerythromycin A
	Et	H	Me	H	Me	H	2,6,10,12-Tetradesmethyl-12-ethylerythromycin A
40	Et	H	Me	H	Et	Me	4,6,10,12-Tetradesmethyl-4,12-diethylerythromycin A
	Et	H	Me	H	H	Me	4,6,10,12-Tetradesmethyl-12-ethylerythromycin A
	Et	Et	Me	H	Me	Et	2,6,10,12-Tetradesmethyl-2,10,12-triethylerythromycin A
	Et	Et	Me	H	Me	H	2,6,10,12-Tetradesmethyl-10,12-diethylerythromycin A
	Et	Et	Me	H	Et	Me	4,6,10,12-Tetradesmethyl-4,10,12-triethylerythromycin A
	Et	Et	Me	H	H	Me	4,6,10,12-Tetradesmethyl-10,12-diethylerythromycin A
45	Et	H	Me	Et	Me	Et	2,6,10,12-Tetradesmethyl-2,6,12-triethylerythromycin A
	Et	H	Me	Et	Me	H	2,6,10,12-Tetradesmethyl-6,12-diethylerythromycin A
	Et	H	Me	Et	H	Me	4,6,10,12-Tetradesmethyl-4,6,12-triethylerythromycin A
	Et	H	Me	Et	H	Me	4,6,10,12-Tetradesmethyl-6,12-diethylerythromycin A
	Et	Et	Me	Et	Me	Et	2,6,10,12-Tetradesmethyl-2,6,10,12-tetraethylerythromycin A
	Et	Et	Me	Et	Me	H	2,6,10,12-Tetradesmethyl-6,10,12-triethylerythromycin A
50	Et	Et	Me	Et	Et	Me	4,6,10,12-Tetradesmethyl-4,6,10,12-tetraethylerythromycin A
	Et	Et	Me	Et	Et	Me	4,6,10,12-Tetradesmethyl-6,10,12-triethylerythromycin A
	Et	Et	Me	Et	H	Me	4,6,10,12-Tetradesmethyl-6,12-diethylerythromycin A
	Et	Et	Me	Et	Me	Et	2,6,10,12-Tetradesmethyl-2,6,10,12-tetraethylerythromycin A
	Et	Et	Me	Et	Et	Me	4,6,10,12-Tetradesmethyl-4,6,10,12-tetraethylerythromycin A
	Et	Et	Me	Et	H	Me	4,6,10,12-Tetradesmethyl-6,10,12-triethylerythromycin A

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	H	H	Me	Me	H	Et	2,4,10,12-Tetradesmethyl-2-ethylerythromycin A
	H	H	Me	Me	H	H	2,4,10,12-Tetradesmethylerythromycin A
	H	Et	Me	Me	H	Et	2,4,10,12-Tetradesmethyl-2,10-diethylerythromycin A
	H	Et	Me	Me	H	H	2,4,10,12-Tetradesmethyl-10-ethylerythromycin A
5	H	H	Me	Me	Et	Et	2,4,10,12-Tetradesmethyl-2,4-diethylerythromycin A
	H	H	Me	Me	Et	H	2,4,10,12-Tetradesmethyl-4-ethylerythromycin A
	H	Et	Me	Me	Et	Et	2,4,10,12-Tetradesmethyl-2,4,10-triethylerythromycin A
	H	Et	Me	Me	Et	H	2,4,10,12-Tetradesmethyl-4,10-diethylerythromycin A
	Et	H	Me	Me	H	Et	2,4,10,12-Tetradesmethyl-2,12-diethylerythromycin A
10	Et	H	Me	Me	H	H	2,4,10,12-Tetradesmethyl-12-ethylerythromycin A
	Et	Et	Me	Me	H	Et	2,4,10,12-Tetradesmethyl-2,10,12-triethylerythromycin A
	Et	Et	Me	Me	H	H	2,4,10,12-Tetradesmethyl-10,12-diethylerythromycin A
	Et	H	Me	Me	Et	Et	2,4,10,12-Tetradesmethyl-2,4,12-triethylerythromycin A
	Et	H	Me	Me	Et	H	2,4,10,12-Tetradesmethyl-4,12-diethylerythromycin A
15	Et	Et	Me	Me	Et	Et	2,4,10,12-Tetradesmethyl-2,4,10,12-tetraethylerythromycin A
	Et	Et	Me	Me	Et	H	2,4,10,12-Tetradesmethyl-4,10,12-triethylerythromycin A
	H	Me	H	H	Me	Et	2,6,8,12-Tetradesmethyl-2-ethylerythromycin A
	H	Me	H	H	Me	H	2,6,8,12-Tetradesmethylerythromycin A
20	H	Me	H	H	Et	Me	4,6,8,12-Tetradesmethyl-4-ethylerythromycin A
	H	Me	H	H	H	Me	4,6,8,12-Tetradesmethylerythromycin A
	H	Me	Et	H	Me	Et	2,6,8,12-Tetradesmethyl-2,8-diethylerythromycin A
	H	Me	Et	H	Me	H	2,6,8,12-Tetradesmethyl-8-ethylerythromycin A
	H	Me	Et	H	Et	Me	4,6,8,12-Tetradesmethyl-4,8-diethylerythromycin A
	H	Me	Et	H	H	Me	4,6,8,12-Tetradesmethyl-8-ethylerythromycin A
25	H	Me	H	Et	Me	Et	2,6,8,12-Tetradesmethyl-2,6-diethylerythromycin A
	H	Me	H	Et	Me	H	2,6,8,12-Tetradesmethyl-6-ethylerythromycin A
	H	Me	H	Et	Et	Me	4,6,8,12-Tetradesmethyl-4,6-diethylerythromycin A
	H	Me	H	Et	H	Me	4,6,8,12-Tetradesmethyl-6-ethylerythromycin A
	H	Me	Et	Et	Me	Et	2,6,8,12-Tetradesmethyl-2,6,8-triethylerythromycin A
30	H	Me	Et	Et	Me	H	2,6,8,12-Tetradesmethyl-6,8-diethylerythromycin A
	H	Me	Et	Et	Et	Me	4,6,8,12-Tetradesmethyl-4,6,8-triethylerythromycin A
	H	Me	Et	Et	H	Me	4,6,8,12-Tetradesmethyl-6,8-diethylerythromycin A
	Et	Me	H	H	Me	Et	2,6,8,12-Tetradesmethyl-2,12-diethylerythromycin A
	Et	Me	H	H	Me	H	2,6,8,12-Tetradesmethyl-12-ethylerythromycin A
35	Et	Me	H	H	Et	Me	4,6,8,12-Tetradesmethyl-4,12-diethylerythromycin A
	Et	Me	H	H	H	Me	4,6,8,12-Tetradesmethyl-12-ethylerythromycin A
	Et	Me	Et	H	Me	Et	2,6,8,12-Tetradesmethyl-2,8,12-triethylerythromycin A
	Et	Me	Et	H	Me	H	2,6,8,12-Tetradesmethyl-8,12-diethylerythromycin A
	Et	Me	Et	H	Et	Me	4,6,8,12-Tetradesmethyl-4,8,12-triethylerythromycin A
40	Et	Me	Et	H	H	Me	4,6,8,12-Tetradesmethyl-8,12-diethylerythromycin A
	Et	Me	H	Et	Me	Et	2,6,8,12-Tetradesmethyl-2,6,12-triethylerythromycin A
	Et	Me	H	Et	Me	H	2,6,8,12-Tetradesmethyl-6,12-diethylerythromycin A
	Et	Me	H	Et	Et	Me	4,6,8,12-Tetradesmethyl-4,6,12-triethylerythromycin A
	Et	Me	H	Et	H	Me	4,6,8,12-Tetradesmethyl-6,12-diethylerythromycin A
45	Et	Me	Et	Et	Me	Et	2,6,8,12-Tetradesmethyl-2,6,8,12-tetraethylerythromycin A
	Et	Me	Et	Et	Me	H	2,6,8,12-Tetradesmethyl-6,8,12-triethylerythromycin A
	Et	Me	Et	Et	Et	Me	4,6,8,12-Tetradesmethyl-4,6,8,12-tetraethylerythromycin A
	Et	Me	Et	Et	H	Me	4,6,8,12-Tetradesmethyl-6,8,12-triethylerythromycin A
50	H	Me	Me	H	H	Et	2,4,6,12-Tetradesmethyl-2-ethylerythromycin A
	H	Me	Me	H	H	H	2,4,6,12-Tetradesmethylerythromycin A
	H	Me	Me	Et	H	Et	2,4,6,12-Tetradesmethyl-2,6-diethylerythromycin A
	H	Me	Me	Et	H	H	2,4,6,12-Tetradesmethyl-6-ethylerythromycin A
	H	Me	Me	H	Et	Et	2,4,6,12-Tetradesmethyl-2,4-diethylerythromycin A
	H	Me	Me	H	Et	H	2,4,6,12-Tetradesmethyl-4-ethylerythromycin A
55	H	Me	Me	Et	Et	Et	2,4,6,12-Tetradesmethyl-2,4,6-triethylerythromycin A
	H	Me	Me	Et	Et	H	2,4,6,12-Tetradesmethyl-4,6-diethylerythromycin A

5	Et	Me	Me	H	H	Et	2,4,6,12-Tetradesmethyl-2,12-diethylerythromycin A
	Et	Me	Me	H	H	H	2,4,6,12-Tetradesmethyl-12-ethylerythromycin A
	Et	Me	Me	Et	H	Et	2,4,6,12-Tetradesmethyl-2,6,12-triethylerythromycin A
	Et	Me	Me	Et	H	H	2,4,6,12-Tetradesmethyl-6,12-diethylerythromycin A
	Et	Me	Me	H	Et	Et	2,4,6,12-Tetradesmethyl-2,4,12-triethylerythromycin A
	Et	Me	Me	H	Et	H	2,4,6,12-Tetradesmethyl--diethylerythromycin A
	Et	Me	Me	Et	Et	Et	2,4,6,12-Tetradesmethyl-2,4,6,12-tetraethylerythromycin A
	Et	Me	Me	Et	Et	H	2,4,6,12-Tetradesmethyl-4,6,12-triethylerythromycin A
10	Me	H	H	H	Me	Et	2,6,8,10-Tetradesmethyl-2-ethylerythromycin A
	Me	H	H	H	Me	H	2,6,8,10-Tetradesmethylerythromycin A
	Me	H	H	H	Et	Me	4,6,8,10-Tetradesmethyl-4-ethylerythromycin A
	Me	H	H	H	H	Me	4,6,8,10-Tetradesmethylerythromycin A
	Me	H	Et	H	Me	Et	2,6,8,10-Tetradesmethyl-2,8-diethylerythromycin A
15	Me	H	Et	H	Me	H	2,6,8,10-Tetradesmethyl-8-ethylerythromycin A
	Me	H	Et	H	Et	Me	4,6,8,10-Tetradesmethyl-4,8-diethylerythromycin A
	Me	H	Et	H	H	Me	4,6,8,10-Tetradesmethyl-8-ethylerythromycin A
	Me	H	H	Et	Me	Et	2,6,8,10-Tetradesmethyl-2,6-diethylerythromycin A
	Me	H	H	Et	Me	H	2,6,8,10-Tetradesmethyl-6-ethylerythromycin A
20	Me	H	H	Et	Et	Me	4,6,8,10-Tetradesmethyl-4,6-diethylerythromycin A
	Me	H	H	Et	H	Me	4,6,8,10-Tetradesmethyl-6-ethylerythromycin A
	Me	H	Et	Et	Me	Et	2,6,8,10-Tetradesmethyl-2,6,8-triethylerythromycin A
	Me	H	Et	Et	Me	H	2,6,8,10-Tetradesmethyl-6,8-diethylerythromycin A
	Me	H	Et	Et	Et	Me	4,6,8,10-Tetradesmethyl-4,6,8-triethylerythromycin A
25	Me	H	Et	Et	H	Me	4,6,8,10-Tetradesmethyl-6,8-diethylerythromycin A
	Me	Et	H	H	Me	Et	2,6,8,10-Tetradesmethyl-2,10-diethylerythromycin A
	Me	Et	H	H	Me	H	2,6,8,10-Tetradesmethyl-10-ethylerythromycin A
	Me	Et	H	H	Et	Me	4,6,8,10-Tetradesmethyl-4,10-diethylerythromycin A
	Me	Et	H	H	H	Me	4,6,8,10-Tetradesmethyl-10-ethylerythromycin A
30	Me	Et	Et	H	Me	Et	2,6,8,10-Tetradesmethyl-2,8,10-triethylerythromycin A
	Me	Et	Et	H	Me	H	2,6,8,10-Tetradesmethyl-8,10-diethylerythromycin A
	Me	Et	Et	H	Et	Me	4,6,8,10-Tetradesmethyl-4,8,10-triethylerythromycin A
	Me	Et	Et	H	H	Me	4,6,8,10-Tetradesmethyl-8,10-diethylerythromycin A
	Me	Et	H	Et	Me	Et	2,6,8,10-Tetradesmethyl-2,6,10-triethylerythromycin A
35	Me	Et	H	Et	Me	H	2,6,8,10-Tetradesmethyl-6,10-diethylerythromycin A
	Me	Et	H	Et	Et	Me	4,6,8,10-Tetradesmethyl-4,6,10-triethylerythromycin A
	Me	Et	H	Et	H	Me	4,6,8,10-Tetradesmethyl-6,10-diethylerythromycin A
	Me	Et	Et	Et	Me	Et	2,6,8,10-Tetradesmethyl-2,6,8,10-tetraethylerythromycin A
	Me	Et	Et	Et	Me	H	2,6,8,10-Tetradesmethyl-6,8,10-triethylerythromycin A
40	Me	Et	Et	Et	Et	Me	4,6,8,10-Tetradesmethyl-4,6,8,10-tetraethylerythromycin A
	Me	Et	Et	Et	H	Me	4,6,8,10-Tetradesmethyl-6,8,10-triethylerythromycin A
	Me	H	H	Me	H	Et	2,4,8,10-Tetradesmethyl-2-ethylerythromycin A
	Me	H	H	Me	H	H	2,4,8,10-Tetradesmethylerythromycin A
	Me	H	Et	Me	H	Et	2,4,8,10-Tetradesmethyl-2,8-diethylerythromycin A
45	Me	H	Et	Me	H	H	2,4,8,10-Tetradesmethyl-8-ethylerythromycin A
	Me	H	H	Me	Et	Et	2,4,8,10-Tetradesmethyl-2,4-diethylerythromycin A
	Me	H	H	Me	Et	H	2,4,8,10-Tetradesmethyl-4-ethylerythromycin A
	Me	H	Et	Me	Et	Et	2,4,8,10-Tetradesmethyl-2,4,8-triethylerythromycin A
	Me	H	Et	Me	Et	H	2,4,8,10-Tetradesmethyl-4,8-diethylerythromycin A
50	Me	Et	H	Me	H	Et	2,4,8,10-Tetradesmethyl-2,10-diethylerythromycin A
	Me	Et	H	Me	H	H	2,4,8,10-Tetradesmethyl-10-ethylerythromycin A
	Me	Et	Et	Me	H	Et	2,4,8,10-Tetradesmethyl-2,8,10-triethylerythromycin A
	Me	Et	Et	Me	H	H	2,4,8,10-Tetradesmethyl-8,10-diethylerythromycin A
	Me	Et	H	Me	Et	Et	2,4,8,10-Tetradesmethyl-2,4,10-triethylerythromycin A
55	Me	Et	H	Me	Et	H	2,4,8,10-Tetradesmethyl-4,10-diethylerythromycin A
	Me	Et	Et	Me	Et	Et	2,4,8,10-Tetradesmethyl-2,4,8,10-tetraethylerythromycin A
	Me	Et	Et	Me	Et	H	2,4,8,10-Tetradesmethyl-4,8,10-triethylerythromycin A

5	Me	Me	H	H	H	Et	2,4,6,8-Tetradesmethyl-2-ethylerythromycin A
	Me	Me	H	H	H	H	2,4,6,8-Tetradesmethylerythromycin A
	Me	Me	H	Et	H	Et	2,4,6,8-Tetradesmethyl-2,6-diethylerythromycin A
	Me	Me	H	Et	H	H	2,4,6,8-Tetradesmethyl-6-ethylerythromycin A
	Me	Me	H	H	Et	Et	2,4,6,8-Tetradesmethyl-2,4-diethylerythromycin A
	Me	Me	H	H	Et	H	2,4,6,8-Tetradesmethyl-4-ethylerythromycin A
	Me	Me	H	Et	Et	Et	2,4,6,8-Tetradesmethyl-2,4,6-triethylerythromycin A
	Me	Me	H	Et	Et	H	2,4,6,8-Tetradesmethyl-4,6-diethylerythromycin A
10	Me	Me	Et	H	H	Et	2,4,6,8-Tetradesmethyl-2,8-diethylerythromycin A
	Me	Me	Et	H	H	H	2,4,6,8-Tetradesmethyl-8-ethylerythromycin A
	Me	Me	Et	Et	H	Et	2,4,6,8-Tetradesmethyl-2,6,8-triethylerythromycin A
	Me	Me	Et	Et	H	H	2,4,6,8-Tetradesmethyl-6,8-diethylerythromycin A
15	Me	Me	Et	H	Et	Et	2,4,6,8-Tetradesmethyl-2,4,8-triethylerythromycin A
	Me	Me	Et	H	Et	H	2,4,6,8-Tetradesmethyl-4,8-diethylerythromycin A
	Me	Me	Et	Et	Et	Et	2,4,6,8-Tetradesmethyl-2,4,6,8-tetraethylerythromycin A
	Me	Me	Et	Et	Et	H	2,4,6,8-Tetradesmethyl-4,6,8-triethylerythromycin A

## E. Five Changes

20	H	H	H	H	H	Me	4,6,8,10,12-Pentadesmethylerythromycin A
	Et	H	H	H	H	Me	4,6,8,10,12-Pentadesmethyl-12-ethylerythromycin A
	H	Et	H	H	H	Me	4,6,8,10,12-Pentadesmethyl-10-ethylerythromycin A
	H	H	Et	H	H	Me	4,6,8,10,12-Pentadesmethyl-8-ethylerythromycin A
25	H	H	H	Et	H	Me	4,6,8,10,12-Pentadesmethyl-6-ethylerythromycin A
	H	H	H	H	Et	Me	4,6,8,10,12-Pentadesmethyl-4-ethylerythromycin A
	Et	Et	H	H	H	Me	4,6,8,10,12-Pentadesmethyl-10,12-diethylerythromycin A
	Et	H	Et	H	H	Me	4,6,8,10,12-Pentadesmethyl-8,12-diethylerythromycin A
	Et	H	H	Et	H	Me	4,6,8,10,12-Pentadesmethyl-6,12-diethylerythromycin A
	Et	H	H	H	Et	Me	4,6,8,10,12-Pentadesmethyl-4,12-diethylerythromycin A
30	H	Et	Et	H	H	Me	4,6,8,10,12-Pentadesmethyl-8,10-diethylerythromycin A
	H	Et	H	Et	H	Me	4,6,8,10,12-Pentadesmethyl-6,10-diethylerythromycin A
	H	Et	H	H	Et	Me	4,6,8,10,12-Pentadesmethyl-4,10-diethylerythromycin A
	H	H	Et	Et	H	Me	4,6,8,10,12-Pentadesmethyl-6,8-diethylerythromycin A
	H	H	Et	H	Et	Me	4,6,8,10,12-Pentadesmethyl-4,8-diethylerythromycin A
	H	H	H	Et	Et	Me	4,6,8,10,12-Pentadesmethyl-4,6-diethylerythromycin A
35	Et	Et	Et	H	H	Me	4,6,8,10,12-Pentadesmethyl-8,10,12-triethylerythromycin A
	Et	Et	H	Et	H	Me	4,6,8,10,12-Pentadesmethyl-6,10,12-triethylerythromycin A
	Et	Et	H	H	Et	Me	4,6,8,10,12-Pentadesmethyl-4,10,12-triethylerythromycin A
	Et	H	Et	Et	H	Me	4,6,8,10,12-Pentadesmethyl-6,8,12-triethylerythromycin A
	Et	H	Et	H	Et	Me	4,6,8,10,12-Pentadesmethyl-4,8,12-triethylerythromycin A
	Et	H	H	Et	Et	Me	4,6,8,10,12-Pentadesmethyl-4,6,12-triethylerythromycin A
40	H	Et	Et	Et	H	Me	4,6,8,10,12-Pentadesmethyl-6,8,10-triethylerythromycin A
	H	Et	Et	H	Et	Me	4,6,8,10,12-Pentadesmethyl-4,8,10-triethylerythromycin A
	H	Et	H	Et	Et	Me	4,6,8,10,12-Pentadesmethyl-4,6,10-triethylerythromycin A
	H	H	Et	Et	Et	Me	4,6,8,10,12-Pentadesmethyl-4,6,8-triethylerythromycin A
	Et	Et	Et	Et	H	Me	4,6,8,10,12-Pentadesmethyl-6,8,10,12-tetraethylerythromycin A
	Et	Et	Et	H	Et	Me	4,6,8,10,12-Pentadesmethyl-4,8,10,12-tetraethylerythromycin A
45	Et	Et	H	Et	Et	Me	4,6,8,10,12-Pentadesmethyl-4,6,10,12-tetraethylerythromycin A
	Et	H	Et	Et	Et	Me	4,6,8,10,12-Pentadesmethyl-4,6,8,12-tetraethylerythromycin A
	H	Et	Et	Et	Et	Me	4,6,8,10,12-Pentadesmethyl-4,6,8,10-tetraethylerythromycin A
	Et	Et	Et	Et	Et	Me	4,6,8,10,12-Pentadesmethyl-4,6,8,10,12-pentaethylerythromycin A
	H	H	H	H	Me	H	2,6,8,10,12-Pentadesmethylerythromycin A
	Et	H	H	H	Me	H	2,6,8,10,12-Pentadesmethyl-12-ethylerythromycin A
50	H	Et	H	H	Me	H	2,6,8,10,12-Pentadesmethyl-10-ethylerythromycin A
	H	H	Et	H	Me	H	2,6,8,10,12-Pentadesmethyl-8-ethylerythromycin A
	H	H	H	Et	Me	H	2,6,8,10,12-Pentadesmethyl-6-ethylerythromycin A
	H	H	H	H	Me	Et	2,6,8,10,12-Pentadesmethyl-2-ethylerythromycin A

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5	Et	Et	H	H	Me	H	2,6,8,10,12-Pentadesmethyl-10,12-diethylerythromycin A
	Et	H	Et	H	Me	H	2,6,8,10,12-Pentadesmethyl-8,12-diethylerythromycin A
	Et	H	H	Et	Me	H	2,6,8,10,12-Pentadesmethyl-4,12-diethylerythromycin A
	Et	H	H	H	Me	Et	2,6,8,10,12-Pentadesmethyl-2,12-diethylerythromycin A
	H	Et	Et	H	Me	H	2,6,8,10,12-Pentadesmethyl-8,10-diethylerythromycin A
10	H	Et	H	Et	Me	H	2,6,8,10,12-Pentadesmethyl-6,10-diethylerythromycin A
	H	Et	H	H	Me	Et	2,6,8,10,12-Pentadesmethyl-2,10-diethylerythromycin A
	H	H	Et	Et	Me	H	2,6,8,10,12-Pentadesmethyl-6,8-diethylerythromycin A
	H	H	Et	H	Me	Et	2,6,8,10,12-Pentadesmethyl-2,8-diethylerythromycin A
	H	H	H	Et	Me	Et	2,6,8,10,12-Pentadesmethyl-2,6-diethylerythromycin A
15	Et	Et	Et	H	Me	H	2,6,8,10,12-Pentadesmethyl-8,10,12-triethylerythromycin A
	Et	Et	H	Et	Me	H	2,6,8,10,12-Pentadesmethyl-6,10,12-triethylerythromycin A
	Et	Et	H	H	Me	Et	2,6,8,10,12-Pentadesmethyl-2,10,12-triethylerythromycin A
	Et	H	Et	Et	Me	H	2,6,8,10,12-Pentadesmethyl-6,8,12-triethylerythromycin A
	Et	H	Et	H	Me	Et	2,6,8,10,12-Pentadesmethyl-2,8,12-triethylerythromycin A
20	Et	H	H	Et	Me	Et	2,6,8,10,12-Pentadesmethyl-2,6,12-triethylerythromycin A
	H	Et	Et	Et	Me	H	2,6,8,10,12-Pentadesmethyl-6,8,10-triethylerythromycin A
	H	Et	Et	H	Me	Et	2,6,8,10,12-Pentadesmethyl-2,8,10-triethylerythromycin A
	H	Et	H	Et	Me	Et	2,6,8,10,12-Pentadesmethyl-2,6,10-triethylerythromycin A
	H	H	Et	Et	Me	Et	2,6,8,10,12-Pentadesmethyl-2,6,8-triethylerythromycin A
25	Et	Et	Et	Et	Me	H	2,6,8,10,12-Pentadesmethyl-6,8,10,12-tetraethylerythromycin A
	Et	Et	Et	H	Me	Et	2,6,8,10,12-Pentadesmethyl-2,8,10,12-tetraethylerythromycin A
	Et	Et	H	Et	Me	Et	2,6,8,10,12-Pentadesmethyl-2,6,10,12-tetraethylerythromycin A
	AEt	H	Et	Et	Me	Et	2,6,8,10,12-Pentadesmethyl-2,6,,8,12-tetraethylerythromycin A
	H	Et	Et	Et	Me	Et	2,6,8,10,12-Pentadesmethyl-2,6,8,10-tetraethylerythromycin A
30	Et	Et	Et	Et	Me	Et	2,6,8,10,12-Pentadesmethyl-2,6,,8,10,12-pentaethylerythromycin A
	H	H	H	Me	H	H	2,4,8,10,12-Pentadesmethylerythromycin A
	Et	H	H	Me	H	H	2,4,8,10,12-Pentadesmethyl-12-ethylerythromycin A
	H	Et	H	Me	H	H	2,4,8,10,12-Pentadesmethyl-10-ethylerythromycin A
	H	H	Et	Me	H	H	2,4,8,10,12-Pentadesmethyl-8-ethylerythromycin A
35	H	H	H	Me	Et	H	2,4,8,10,12-Pentadesmethyl-4-ethylerythromycin A
	H	H	H	Me	H	Et	2,4,8,10,12-Pentadesmethyl-2-ethylerythromycin A
	Et	Et	H	Me	H	H	2,4,8,10,12-Pentadesmethyl-10,12-diethylerythromycin A
	Et	H	Et	Me	H	H	2,4,8,10,12-Pentadesmethyl-8,12-diethylerythromycin A
	Et	H	H	Me	Et	H	2,4,8,10,12-Pentadesmethyl-4,12-diethylerythromycin A
40	Et	H	H	Me	H	Et	2,4,8,10,12-Pentadesmethyl-2,12-diethylerythromycin A
	H	Et	Et	Me	H	H	2,4,8,10,12-Pentadesmethyl-8,10-diethylerythromycin A
	H	Et	H	Me	Et	H	2,4,8,10,12-Pentadesmethyl-4,10-diethylerythromycin A
	H	Et	H	Me	H	Et	2,4,8,10,12-Pentadesmethyl-2,10-diethylerythromycin A
	H	H	Et	Me	Et	H	2,4,8,10,12-Pentadesmethyl-4,8-diethylerythromycin A
45	H	H	H	Me	Et	Et	2,4,8,10,12-Pentadesmethyl-2,4-diethylerythromycin A
	Et	Et	Et	Me	H	H	2,4,8,10,12-Pentadesmethyl-8,10,12-triethylerythromycin A
	Et	Et	H	Me	Et	H	2,4,8,10,12-Pentadesmethyl-4,10,12-triethylerythromycin A
	Et	Et	H	Me	H	Et	2,4,8,10,12-Pentadesmethyl-2,10,12-triethylerythromycin A
	Et	H	Et	Me	Et	H	2,4,8,10,12-Pentadesmethyl-4,8,12-triethylerythromycin A
50	Et	H	H	Me	Et	Et	2,4,8,10,12-Pentadesmethyl-2,8,12-triethylerythromycin A
	H	Et	Et	Me	Et	H	2,4,8,10,12-Pentadesmethyl-2,4,12-triethylerythromycin A
	H	Et	Et	Me	H	Et	2,4,8,10,12-Pentadesmethyl-4,8,10-triethylerythromycin A
	H	Et	H	Me	Et	Et	2,4,8,10,12-Pentadesmethyl-2,8,10-triethylerythromycin A
	H	H	Et	Me	Et	Et	2,4,8,10,12-Pentadesmethyl-2,4,10-triethylerythromycin A
55	Et	Et	Et	Me	Et	H	2,4,8,10,12-Pentadesmethyl-4,8,10,12-tetraethylerythromycin A
	Et	Et	Et	Me	H	Et	2,4,8,10,12-Pentadesmethyl-2,8,10,12-tetraethylerythromycin A
	Et	Et	H	Me	Et	Et	2,4,8,10,12-Pentadesmethyl-2,4,10,12-tetraethylerythromycin A
	Et	H	Et	Me	Et	Et	2,4,8,10,12-Pentadesmethyl-2,4,8,12-tetraethylerythromycin A

	H	Et	Et	Me	Et	Et	2,4,8,10,12-Pentadesmethyl-2,4,8,10-tetraethylerythromycin A
	Et	Et	Et	Me	Et	Et	2,4,8,10,12-Pentadesmethyl-2,4,8,10,12-pentaethylerythromycin A
	H	H	Me	H	H	H	2,4,6,10,12-Pentadesmethylerythromycin
	Et	H	Me	H	H	H	2,4,6,10,12-Pentadesmethyl-12-ethylerythromycin A
5	H	Et	Me	H	H	H	2,4,6,10,12-Pentadesmethyl-10-ethylerythromycin A
	H	H	Me	Et	H	H	2,4,6,10,12-Pentadesmethyl-6-ethylerythromycin A
	H	H	Me	H	Et	H	2,4,6,10,12-Pentadesmethyl-4-ethylerythromycin A
	H	H	Me	H	H	Et	2,4,6,10,12-Pentadesmethyl-2-ethylerythromycin A
10	Et	Et	Me	H	H	H	2,4,6,10,12-Pentadesmethyl-10,12-diethylerythromycin A
	Et	H	Me	Et	H	H	2,4,6,10,12-Pentadesmethyl-6,12-diethylerythromycin A
	Et	H	Me	H	Et	H	2,4,6,10,12-Pentadesmethyl-4,12-diethylerythromycin A
	Et	H	Me	H	H	Et	2,4,6,10,12-Pentadesmethyl-2,12-diethylerythromycin A
	H	Et	Me	Et	H	H	2,4,6,10,12-Pentadesmethyl-6,10-diethylerythromycin A
15	H	Et	Me	H	Et	H	2,4,6,10,12-Pentadesmethyl-4,10-diethylerythromycin A
	H	Et	Me	H	H	Et	2,4,6,10,12-Pentadesmethyl-2,10-diethylerythromycin A
	H	H	Me	Et	Et	H	2,4,6,10,12-Pentadesmethyl-4,6-diethylerythromycin A
	H	H	Me	Et	H	Et	2,4,6,10,12-Pentadesmethyl-2,6-diethylerythromycin A
	H	H	Me	H	Et	Et	2,4,6,10,12-Pentadesmethyl-2,4-diethylerythromycin A
20	Et	Et	Me	Et	H	H	2,4,6,10,12-Pentadesmethyl-6,10,12-triethylerythromycin A
	Et	Et	Me	H	Et	H	2,4,6,10,12-Pentadesmethyl-4,10,12-triethylerythromycin A
	Et	Et	Me	H	H	Et	2,4,6,10,12-Pentadesmethyl-2,10,12-triethylerythromycin A
	Et	H	Me	Et	Et	H	2,4,6,10,12-Pentadesmethyl-4,6,12-triethylerythromycin A
	Et	H	Me	Et	H	Et	2,4,6,10,12-Pentadesmethyl-2,6,12-triethylerythromycin A
25	Et	H	Me	H	Et	Et	2,4,6,10,12-Pentadesmethyl-2,4,12-triethylerythromycin A
	H	Et	Me	Et	Et	H	2,4,6,10,12-Pentadesmethyl-4,6,10-triethylerythromycin A
	H	Et	Me	Et	H	Et	2,4,6,10,12-Pentadesmethyl-2,6,10-triethylerythromycin A
	H	Et	Me	H	Et	Et	2,4,6,10,12-Pentadesmethyl-2,4,10-triethylerythromycin A
	H	H	Me	Et	Et	Et	2,4,6,10,12-Pentadesmethyl-2,4,6-triethylerythromycin A
30	Et	Et	Me	Et	Et	H	2,4,6,10,12-Pentadesmethyl-4,6,10,12-tetraethylerythromycin A
	Et	Et	Me	Et	H	Et	2,4,6,10,12-Pentadesmethyl-2,6,10,12-tetraethylerythromycin A
	Et	Et	Me	H	Et	Et	2,4,6,10,12-Pentadesmethyl-2,4,10,12-tetraethylerythromycin A
	Et	H	Me	Et	Et	Et	2,4,6,10,12-Pentadesmethyl-2,4,6,12-tetraethylerythromycin A
	H	Et	Me	Et	Et	Et	2,4,6,10,12-Pentadesmethyl-2,4,6,10-tetraethylerythromycin A
35	Et	Et	Me	Et	Et	Et	2,4,6,10,12-Pentadesmethyl-2,4,6,10,12-pentaethylerythromycin A
	H	Me	H	H	H	H	2,4,6,8,12-Pentadesmethylerythromycin A
	Et	Me	H	H	H	H	2,4,6,8,12-Pentadesmethyl-12-ethylerythromycin A
	H	Me	Et	H	H	H	2,4,6,8,12-Pentadesmethyl-8-ethylerythromycin A
	H	Me	H	Et	H	H	2,4,6,8,12-Pentadesmethyl-6-ethylerythromycin A
40	H	Me	H	H	Et	H	2,4,6,8,12-Pentadesmethyl-4-ethylerythromycin A
	H	Me	H	H	H	Et	2,4,6,8,12-Pentadesmethyl-2-ethylerythromycin A
	Et	Me	Et	H	H	H	2,4,6,8,12-Pentadesmethyl-8,12-diethylerythromycin A
	Et	Me	H	Et	H	H	2,4,6,8,12-Pentadesmethyl-6,12-diethylerythromycin A
	Et	Me	H	H	Et	H	2,4,6,8,12-Pentadesmethyl-4,12-diethylerythromycin A
45	Et	Me	H	H	H	Et	2,4,6,8,12-Pentadesmethyl-2,12-diethylerythromycin A
	H	Me	Et	Et	H	H	2,4,6,8,12-Pentadesmethyl-6,8-diethylerythromycin A
	H	Me	Et	H	Et	H	2,4,6,8,12-Pentadesmethyl-4,8-diethylerythromycin A
	H	Me	Et	H	H	Et	2,4,6,8,12-Pentadesmethyl-2,8-diethylerythromycin A
	H	Me	H	Et	Et	H	2,4,6,8,12-Pentadesmethyl-4,6-diethylerythromycin A
50	H	Me	H	Et	H	Et	2,4,6,8,12-Pentadesmethyl-2,6-diethylerythromycin A
	H	Me	H	H	Et	Et	2,4,6,8,12-Pentadesmethyl-2,4-diethylerythromycin A
	Et	Me	Et	Et	H	H	2,4,6,8,12-Pentadesmethyl-6,8,12-triethylerythromycin A
	Et	Me	Et	H	Et	H	2,4,6,8,12-Pentadesmethyl-4,8,12-triethylerythromycin A
	Et	Me	Et	H	H	Et	2,4,6,8,12-Pentadesmethyl-2,8,12-triethylerythromycin A
	Et	Me	H	Et	Et	H	2,4,6,8,12-Pentadesmethyl-4,6,12-triethylerythromycin A
55	Et	Me	H	Et	H	Et	2,4,6,8,12-Pentadesmethyl-2,6,12-triethylerythromycin A
	Et	Me	H	H	Et	Et	2,4,6,8,12-Pentadesmethyl-2,4,12-triethylerythromycin A

5	H	Me	Et	Et	Et	H	2,4,6,8,12-Pentadesmethyl-4,6,8-triethylerythromycin A
	H	Me	Et	Et	H	Et	2,4,6,8,12-Pentadesmethyl-2,6,8-triethylerythromycin A
	H	Me	Et	H	Et	Et	2,4,6,8,12-Pentadesmethyl-2,4,8-triethylerythromycin A
	H	Me	H	Et	Et	Et	2,4,6,8,12-Pentadesmethyl-2,4,6-triethylerythromycin A
	Et	Me	Et	Et	Et	H	2,4,6,8,12-Pentadesmethyl-4,6,8-triethylerythromycin A
	Et	Me	Et	Et	H	Et	2,4,6,8,12-Pentadesmethyl-2,6,8,12-tetraethylerythromycin A
	Et	Me	Et	H	Et	Et	2,4,6,8,12-Pentadesmethyl-2,4,8,12-tetraethylerythromycin A
10	Et	Me	H	Et	Et	Et	2,4,6,8,12-Pentadesmethyl-2,4,6,12-tetraethylerythromycin A
	H	Me	Et	Et	Et	Et	2,4,6,8,12-Pentadesmethyl-2,4,6,8-tetraethylerythromycin A
	Et	Me	Et	Et	Et	Et	2,4,6,8,12-Pentadesmethyl-2,4,6,8,12-pentaethylerythromycin A
	Me	H	H	H	H	H	2,4,6,8,10-Pentadesmethylerythromycin A
	Me	Et	H	H	H	H	2,4,6,8,10-Pentadesmethyl-10-ethylerythromycin A
	Me	H	Et	H	H	H	2,4,6,8,10-Pentadesmethyl-8-ethylerythromycin A
	Me	H	H	Et	H	H	2,4,6,8,10-Pentadesmethyl-6-ethylerythromycin A
15	Me	H	H	H	Et	H	2,4,6,8,10-Pentadesmethyl-4-ethylerythromycin A
	Me	H	H	H	H	Et	2,4,6,8,10-Pentadesmethyl-2-ethylerythromycin A
	Me	Et	Et	H	H	H	2,4,6,8,10-Pentadesmethyl-8,10 diethylerythromycin A
	Me	Et	H	Et	H	H	2,4,6,8,10-Pentadesmethyl-6,10 diethylerythromycin A
	Me	Et	H	H	Et	H	2,4,6,8,10-Pentadesmethyl-4,10 diethylerythromycin A
	Me	Et	H	H	H	Et	2,4,6,8,10-Pentadesmethyl-2,10 diethylerythromycin A
	Me	H	Et	Et	H	H	2,4,6,8,10-Pentadesmethyl-6,8-diethylerythromycin A
20	Me	H	Et	H	Et	H	2,4,6,8,10-Pentadesmethyl-4,8-diethylerythromycin A
	Me	H	Et	H	H	Et	2,4,6,8,10-Pentadesmethyl-2,8-diethylerythromycin A
	Me	H	H	Et	Et	H	2,4,6,8,10-Pentadesmethyl-4,6-diethylerythromycin A
	Me	H	H	Et	H	Et	2,4,6,8,10-Pentadesmethyl-2,6-diethylerythromycin A
	Me	H	H	H	Et	Et	2,4,6,8,10-Pentadesmethyl-2,4-diethylerythromycin A
	Me	Et	Et	Et	H	H	2,4,6,8,10-Pentadesmethyl-6,8,10-triethylerythromycin A
	Me	Et	Et	H	Et	H	2,4,6,8,10-Pentadesmethyl-4,8,10-triethylerythromycin A
25	Me	Et	Et	H	Et	H	2,4,6,8,10-Pentadesmethyl-2,8,10-triethylerythromycin A
	Me	Et	H	Et	Et	H	2,4,6,8,10-Pentadesmethyl-4,6,10-triethylerythromycin A
	Me	Et	H	Et	H	Et	2,4,6,8,10-Pentadesmethyl-2,6,10-triethylerythromycin A
	Me	Et	H	H	Et	Et	2,4,6,8,10-Pentadesmethyl-2,4,10-triethylerythromycin A
	Me	H	Et	Et	Et	H	2,4,6,8,10-Pentadesmethyl-4,6,8-triethylerythromycin A
	Me	H	Et	Et	H	Et	2,4,6,8,10-Pentadesmethyl-2,6,8-triethylerythromycin A
	Me	H	Et	H	Et	Et	2,4,6,8,10-Pentadesmethyl-2,4,8-triethylerythromycin A
30	Me	H	H	Et	Et	Et	2,4,6,8,10-Pentadesmethyl-2,4,6-triethylerythromycin A
	Me	Et	Et	Et	Et	H	2,4,6,8,10-Pentadesmethyl-4,6,8,10-tetraethylerythromycin A
	Me	Et	Et	Et	H	Et	2,4,6,8,10-Pentadesmethyl-2,6,8,10-tetraethylerythromycin A
	Me	Et	Et	H	Et	Et	2,4,6,8,10-Pentadesmethyl-2,4,8,10-tetraethylerythromycin A
	Me	Et	H	Et	Et	Et	2,4,6,8,10-Pentadesmethyl-2,4,6,10-tetraethylerythromycin A
	Me	H	Et	Et	Et	Et	2,4,6,8,10-Pentadesmethyl-2,4,6,8-tetraethylerythromycin A
	Me	Et	Et	Et	Et	Et	2,4,6,8,10-Pentadesmethyl-2,4,6,8,10-pentaethylerythromycin A

## F. Six Changes

45	H	H	H	H	H	H	2,4,6,8,10,12-Hexadesmethylerythromycin A
	Et	H	H	H	H	H	2,4,6,8,10,12-Hexadesmethyl-12-ethylerythromycin A
	H	Et	H	H	H	H	2,4,6,8,10,12-Hexadesmethyl-10-ethylerythromycin A
	H	H	Et	H	H	H	2,4,6,8,10,12-Hexadesmethyl-8-ethylerythromycin A
	H	H	H	Et	H	H	2,4,6,8,10,12-Hexadesmethyl-6-ethylerythromycin A
	H	H	H	H	Et	H	2,4,6,8,10,12-Hexadesmethyl-4-ethylerythromycin A
	H	H	H	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2-ethylerythromycin A
50	Et	Et	H	H	H	H	2,4,6,8,10,12-Hexadesmethyl-10,12-diethylerythromycin A
	Et	H	Et	H	H	H	2,4,6,8,10,12-Hexadesmethyl-8,12-diethylerythromycin A
	Et	H	H	Et	H	H	2,4,6,8,10,12-Hexadesmethyl-6,12-diethylerythromycin A
	Et	H	H	H	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,12-diethylerythromycin A
	Et	H	H	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,12-diethylerythromycin A
	Et	H	H	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,12-diethylerythromycin A
	Et	H	H	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,12-diethylerythromycin A

5	H	Et	Et	H	H	H	2,4,6,8,10,12-Hexadesmethyl-8,10-diethylerythromycin A
	H	Et	H	Et	H	H	2,4,6,8,10,12-Hexadesmethyl-6,10-diethylerythromycin A
	H	Et	H	H	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,10-diethylerythromycin A
	H	Et	H	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,10-diethylerythromycin A
	H	H	Et	Et	H	H	2,4,6,8,10,12-Hexadesmethyl-6,8-diethylerythromycin A
	H	H	Et	H	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,8-diethylerythromycin A
	H	H	Et	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,8-diethylerythromycin A
	H	H	H	Et	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,6-diethylerythromycin A
10	H	H	H	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,6-diethylerythromycin A
	H	H	H	H	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4-diethylerythromycin A
	Et	Et	Et	H	H	H	2,4,6,8,10,12-Hexadesmethyl-8,10,12-triethylerythromycin A
	Et	Et	H	Et	H	H	2,4,6,8,10,12-Hexadesmethyl-6,10,12-triethylerythromycin A
15	Et	Et	H	H	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,10,12-triethylerythromycin A
	Et	Et	H	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,10,12-triethylerythromycin A
	Et	H	Et	Et	H	H	2,4,6,8,10,12-Hexadesmethyl-6,8,12-triethylerythromycin A
	Et	H	Et	H	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,8,12-triethylerythromycin A
20	Et	H	Et	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,8,12-triethylerythromycin A
	Et	H	H	Et	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,6,12-triethylerythromycin A
	Et	H	H	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,6,12-triethylerythromycin A
	Et	H	H	H	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,12-triethylerythromycin A
	H	Et	Et	Et	H	H	2,4,6,8,10,12-Hexadesmethyl-6,8,10-triethylerythromycin A
	H	Et	Et	H	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,8,10-triethylerythromycin A
25	H	Et	Et	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,8,10-triethylerythromycin A
	H	Et	H	Et	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,6,10-triethylerythromycin A
	H	Et	H	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,6,10-triethylerythromycin A
	H	Et	H	H	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,10-triethylerythromycin A
	H	H	Et	Et	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,6,8-triethylerythromycin A
	H	H	Et	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,6,8-triethylerythromycin A
30	H	H	Et	H	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,8-triethylerythromycin A
	H	H	H	Et	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,6-triethylerythromycin A
	Et	Et	Et	Et	H	H	2,4,6,8,10,12-Hexadesmethyl-6,8,10,12-tetraethylerythromycin A
	Et	Et	Et	H	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,8,10,12-tetraethylerythromycin A
35	Et	Et	H	Et	Et	H	2,4,6,8,10,12-Hexadesmethyl-2,8,10,12-tetraethylerythromycin A
	Et	Et	H	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-4,6,10,12-tetraethylerythromycin A
	Et	Et	H	H	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,6,10,12-tetraethylerythromycin A
	Et	H	Et	Et	Et	H	2,4,6,8,10,12-Hexadesmethyl-2,4,10,12-tetraethylerythromycin A
40	Et	H	Et	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-4,6,8,12-tetraethylerythromycin A
	Et	H	Et	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,6,8,12-tetraethylerythromycin A
	Et	H	Et	H	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,8,12-tetraethylerythromycin A
	Et	H	H	Et	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,6,12-tetraethylerythromycin A
	H	Et	Et	Et	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,6,8,10-tetraethylerythromycin A
	H	Et	Et	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,6,8,10-tetraethylerythromycin A
45	H	Et	Et	H	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,8,10-tetraethylerythromycin A
	H	Et	H	Et	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,6,10-tetraethylerythromycin A
	H	H	Et	Et	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,6,8-tetraethylerythromycin A
	Et	Et	Et	Et	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,6,8,10,12-pentaethylerythromycin A
50	Et	Et	Et	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,6,8,10,12-pentaethylerythromycin A
	Et	Et	Et	H	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,8,10,12-pentaethylerythromycin A
	Et	Et	H	Et	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,6,10,12-pentaethylerythromycin A
	Et	H	Et	Et	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,6,8,12-pentaethylerythromycin A
	H	Et	Et	Et	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,6,8,10-pentaethylerythromycin A
	Et	Et	Et	Et	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,6,8,10,12-hexaethylerythromycin

Although in the Examples that follow the AT-encoding DNA fragments from *S.*

55 *hygroscopicus* ATCC 29253, *S. venezuelae* ATCC 15439, and *S. caelestis* NRRL-2821 were



used to replace resident AT-encoding DNA fragments in the eryPKS to yield desmethyl, desmethylethyl, and desmethylhydroxyerythromycins, it is understood that many malonate, ethylmalonate, and hydroxymalonate AT-encoding DNA fragments can be used in place of or in addition to the heterologous malonate, ethylmalonate, and hydroxymalonate-AT DNA fragments described herein to produce the same desmethyl, desmethylethyl, and desmethylhydroxyerythromycin compounds. Examples of DNA fragments encoding malonate-AT domains that can be used in place of or in addition to those specifically described in the Examples below include but are not limited to the DNA fragments encoding AT domains from modules 2, 5, 8, 9, 11, or 12 of the rapamycin PKS genes from *S. hygroscopicus*, the AT domain from module 2 of the PKS responsible the synthesis of methymycin or pikromycin by *S. venezuelae*, the AT domains from modules 3 and 7 of the PKS responsible for the synthesis of tylosin by *S. fradiae*, or the AT domains from modules 1, 2, 3 and 7 of the PKS responsible for the synthesis of spiramycin by *S. ambofaciens*. Examples of DNA fragments encoding ethylmalonate-AT domains that can be used in place of or in addition to those specifically described in the Examples below include but are not limited to the DNA fragments encoding the AT domain from module 5 of the spiramycin PKS genes from *S. ambofaciens*, the AT domain from module 5 of the tylosin PKS genes from *S. fradiae*, and the AT domain from module 5 of the maridomycin PKS genes of *S. hygroscopicus*. Examples of DNA fragments encoding hydroxymalonate-AT domains that can be used in place of or in addition to those specifically described in the Examples below include but are not limited to the DNA fragments encoding the AT domain from module 6 of the spiramycin PKS genes from *S. ambofaciens*, the AT domain from module 6 of the maridomycin PKS genes from *S. hygroscopicus*, and the AT domain from module 6 of the leucomycin PKS genes from *Streptoverticillium kitasatoensis*. Thus the use of any and all DNA fragments encoding malonate, ethylmalonate, and hydroxymalonate-ATs to replace any of the resident DNA fragments encoding methylmalonate-ATs in the eryPKS genes to result in the production of novel derivatives of erythromycin are considered within the scope of the present invention.

Furthermore, those of ordinary skill understand that following the methods described herein for replacement of resident AT-encoding DNA fragments in the eryPKS, the DNA fragments encoding malonate-ATs in *S. hygroscopicus*, *S. venezuelae*, or *S. caelestis*, and ethylmalonate or hydroxymalonate-ATs in *S. caelestis* may be replaced with those AT-encoding DNA fragments from the eryPKS which utilize methylmalonyl CoA as a substrate. As with the eryPKS, all combinations are contemplated, leading to the production of, for example, 13-methylrapamycin, 15-methylrapamycin, 33-methylrapamycin, 13,15-dimethylrapamycin, 13,15,33-trimethylrapamycin, and 10-methylpikromycin.

The methods of the present invention are widely applicable to all erythromycin-producing microorganisms, of which a non-exhaustive list includes *Saccharopolyspora*



species, *Streptomyces griseoplanus*, *Nocardia* sp., *Micromonospora* sp., *Arthrobacter* sp. and *Streptomyces antibioticus*. Of these, *Sac. erythraea* is the most preferred. Other hosts, which normally do not produce erythromycin but into which the erythromycin biosynthesis genes can be introduced by cloning, can also be employed. Such strains include but are not limited to *Streptomyces coelicolor* and *Streptomyces lividans* or *Bacillus subtilis*, as examples. In each of the other erythromycin-producing strains, replacement of the resident AT domains in the erythromycin PKS is conducted by double homologous recombination using cloned eryPKS sequences on both sides of the AT domain to be replaced to effect the switching of the resident AT with a heterologous AT as illustrated in the Examples that follow.

Many other variations of the methods that are illustrated in the Examples that follow will occur to those skilled in the art. For example, whereas the plasmids pUC18, pUC19, pGEM3Zf, and pCS5 were employed in the present invention for the cloning of the LigAT2, venAT, rapAT14, NidAT5, or NidAT6-encoding DNA fragments and construction of the integration vectors, other plasmids, phage, or phagemids including but not limited to pBR322, pACYC184, M13mp18, M13mp19, pGEM7Zf and the like can be used in their place to allow the same constructions to be made. Furthermore, many alternative strategies can be followed for the cloning of the heterologous AT-encoding DNA fragments into integration vectors that enable homologous recombination to occur in corresponding regions of the eryPKS. Examples of alternative strategies include the use of longer or shorter fragments of DNA corresponding to either the AT domains or the flanking sequences, using different restriction sites for the cloning of the AT domains or the adjacent flanking sequences, or changing the sequence of a resident AT-encoding DNA fragment so that it expresses a domain which recognizes malonyl CoA as a substrate rather than methylmalonyl CoA. All such variations are within the scope of the present invention. Similarly, employing alternative strategies to introduce DNA into *Sac. erythraea* or other erythromycin-producing hosts for the purpose of effecting gene exchange to result in the production of novel erythromycins, such as conjugation, transduction or electroporation are also included within the scope of the present invention.

Those skilled in the art also understand that erythromycins B, C and D are naturally occurring forms of erythromycin and therefore would be produced as novel derivatives in *Sac. erythraea* by the modifications disclosed herein. Production of these forms may be further enhanced by inactivation of *eryK* (Stassi, D. et al. *J. Bacteriology*, **175**:182-189, (1993)) to yield erythromycin B derivatives, *eryG* (S. F. Haydock et al. *Mol. Gen. Genet.* **230**:120-128(1991)) to yield erythromycin C derivatives and *eryK* and *eryG* to yield erythromycin D derivatives. Furthermore, in *Sac. erythraea*, 6-deoxy forms of the novel erythromycins A, B, C and D can be generated by inactivation of *eryF* (J. M. Weber et al. *Science* **252**:114-117(1991)) (in addition to those specified above), which encodes the hydroxylase responsible for hydroxylating the C-6 position. In addition, conversion of 6-

deoxy forms of the novel erythromycins A, B, C and D to their corresponding erythromycin A, B, C, and D derivatives may be accomplished by cloning additional copies or by employing other means of overexpression of the *eryF* gene in the production host. Similarly, conversion of novel forms of erythromycins B, C and D to novel forms of erythromycin A may be achieved by expressing or overexpressing *eryK* and/or *eryG* in the production host. The methodologies for generating erythromycins B, C and D and 6-deoxyerythromycins A, B, C and D are well known to those of ordinary skill in the art.

Those skilled in the art also understand that erythronolide B and 3- $\alpha$ -mycarosylerythronolide B are naturally occurring intermediates in the biosynthesis of erythromycin and therefore would be produced as novel intermediates in *Sac. erythraea* by the modifications disclosed herein. Production of these forms may be further enhanced by inactivation of any of the *eryB* genes to yield erythronolide B or *eryC* genes to yield 3- $\alpha$ -L-mycarosylerythronolide B (Weber et al. *J. Bacteriol.* **172**:2372-2383 (1990)) and Haydock et al. *Mol. Gen. Genet.* **230**:120-128 (1991)). Furthermore, 6-deoxy forms of these novel intermediates can be generated by inactivation of *eryF* as described above. The methodologies for generating erythronolide B and 3- $\alpha$ -mycarosylerythronolide B, as well as their 6-deoxy derivatives, are well known to those of ordinary skill in the art.

#### Bacterial Strains, Plasmid Vectors, and Growth Media

The erythromycin-producing microorganism used to practice the following examples of the invention was *Sac. erythraea* ER720 (J.P. DeWitt, *J. Bacteriol.* **164**: 969 (1985)). The host strain for the growth of *E. coli* derived plasmids was DH5 $\alpha$  from GIBCO BRL, Gaithersburg, MD). The *S. hygroscopicus* strain that carries the Lig-PKS cluster is available from the American Type Culture Collection, Bethesda, MD under the accession number ATCC 29253. The *S. venezuelae* strain that carries the venAT domain described herein is available from the American Type Culture Collection, Bethesda, MD under the accession number ATCC 15439.

*E. coli* bacteria carrying pUC18/venAT has been deposited at the Agricultural Research Culture Collection (NRRL), 1815 N. University Street, Peoria, Illinois 61604 U.S.A., as of December 23, 1996, under the terms of the Budapest Treaty and will be maintained for a period of thirty (30) years from the date of deposit, or for five (5) years after the last request for the deposit, or for the enforceable period of the U.S. patent, whichever is longer. The deposit and any other deposited material described herein are provided for convenience only, and are not required to practice the present invention in view of the teachings provided herein. The DNA sequence in all of the deposited material is incorporated herein by reference. *E. coli* bacteria carrying pUC18/venAT was accorded NRRL Deposit No B-21652.

Plasmid pUC18 and pUC19 can be obtained from GIBCO BRL. Plasmid pCS5, a multifunctional vector for integrative transformation of *Sac. erythraea* is described in Vara, *et al.*, *J. Bacteriology*, **171**:5872-5881 (1989) and is referred to therein as pWHM3. Cosmid pNJ1 is described in Tuan, *et al.*, *Gene*, **90**: 21-29 (1990).

5        *Sac. erythraea* was grown for protoplast formation and routine liquid culture in 50 mL of SGGP medium (Yamamoto, *et al.*, *J. Antibiotic*. **39**:1304 (1986)), supplemented with 10 µg of thiostrepton/mL for plasmid selection where appropriate.

#### Reagents and General Methods

10        Commercially available reagents were used to make compounds, plasmids and genetic variants of the present invention, including butyric acid, ampicillin, thiostrepton, restriction endonucleases, T4-DNA ligase, and calf intestine alkaline phosphatase. The nucleotide sequence of the *eryA* genes from *Sac. erythraea* has been deposited in the GenBank database under the accession numbers M63676 and M63677 and are publicly  
15        available.

Standard molecular biology procedures (Maniatis *et al.*, *supra*) were used for the construction and characterization of replacement plasmids. Plasmid DNA was routinely isolated by the alkaline lysis method (H. C. Birnboim and J. Doly, 1979 *Nucleic Acids Res.* **7**: 1513) or with QIAprep Spin Plasmid kit (Qiagen, Inc., Chatsworth, CA) according to the  
20        manufacturers instructions. Restriction fragments were recovered from 0.8-1% agarose gels with Prep-A-Gene (BioRad). The products of ligation for each step of the plasmid constructions were used to transform the intermediate host, *E. coli* DH5α (GIBCO BRL), which was cultured in the presence of ampicillin to select for host cells carrying recombinant plasmids. Selection for insert DNA with X-gal was used where appropriate. Typically, LB  
25        plates contain 30 mL of LB agar (Maniatis *et al.*, *supra*). Plasmid DNAs were isolated from individual transformants that had been grown in liquid culture and characterized with respect to known restriction sites. DNA sequence determination was by cycle sequencing (*fmoI* DNA Sequencing System, Promega Corp. Madison, WI) according to the manufacturer's instructions.

30        SCM medium consist of 20 g Soytone, 15 g Soluble Starch, 10.5 g MOPS, 1.5 g Yeast Extract and 0.1 g CaCl<sub>2</sub> per liter of distilled H<sub>2</sub>O. SGGP medium is described in Yamamoto, *et al.*, 1986, *J. Antibiotic*. **39**:1304. PM buffer (per liter) is 200 g sucrose, 0.25 g K<sub>2</sub>SO<sub>4</sub> in 890 mL H<sub>2</sub>O, with the addition after sterilization of 100 mL 0.25 M TES, pH 7.2, 2 mL trace elements solution (Hopwood, *et al.*, 1985, *Genetic Manipulation of Streptomyces*  
35        A Laboratory Manual, The John Innes Foundation), 0.08 mL 2.5 M CaCl<sub>2</sub>, 10 mL 0.5% KH<sub>2</sub>PO<sub>4</sub>, 2 mL 2.5 M MgCl<sub>2</sub>.

Integrative transformation of *Sac. erythraea* protoplasts, and routine growth and sporulation were carried out according to procedures described in Donadio, *et al.*, 1991,

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Science 115:97; Weber and Losick, 1988, Gene 68:173; and Yamamoto, *et al.*, 1986, J. Antibiotic. 39:1304.

Oligo primers used in the PCR amplifications and described in the Examples below are as follows:

5

5' -ATCTACACSTCSGGCACACSGGCAAGCCSAAGGG-3'	SEQ ID NO:3
5' -CTSAAGGCSGGCGGCGCSTACGTSCCSATCGACCC)-3'	SEQ ID NO:4
5' -CGCGAATTCCCTAGGCTGGCGGTGATGTTCA-3'	SEQ ID NO:5
5' -GCCGGATCCATGCATACGTCCGGCAGGGAGGTAC-3'	SEQ ID NO:6
5' -GCTCGAATTCGCTGGTCCGCGTGACCT-3'	SEQ ID NO:7
5' -GACGGATCCGGCCCTAGGCTGCGCCCGGCTCG-3'	SEQ ID NO:8
5' -TTGGGATCCTATGCATTCCAGCGCGAGCGC-3'	SEQ ID NO:9
5' -GAGAAGCTTGGCGCGACTTGCCCGCT-3'	SEQ ID NO:10
5' -TTT'TTTTAAGCTTGGTACCTGCTCACCGCAACACCG-3'	SEQ ID NO:11
5' -TTT'TTTTGGATCCCTGCAGCCTAGGGTCGGAGGGTACTGCGCGGT-3'	SEQ ID NO:12
5' -TTT'TTTTCTGCAGTATGCATTCCAGGGCAAGCGGTTCCT-3'	SEQ ID NO:13
5' -TTT'TTTTGAATTCACGCGTTGCCCGCGGCGTAGGCGC-3'	SEQ ID NO:14
5' -GATCGAATTCCTTAGGACGGCAGTCCGTGCTCAC-3'	SEQ ID NO:15
5' -GATCGGATCCATGCATACGTCCGAAGGTTCGACCCG-3'	SEQ ID NO:16
5' -TTCGAAGAATTCCTTAGGGTTGCCCTTCCTGTTCGAC-3'	SEQ ID NO:17
5' -TTCGAAAAGCTTATGCATAGACGGCAGATCCACCG-3'	SEQ ID NO:18
5' -CGGTSAGTCSAACATCGG-3'	SEQ ID NO:19
5' -GCRATCTCRCCCTGCGARTG-3'	SEQ ID NO:20
5' -GAGAGAGGAACCAACGCGCACGTGATCGTCAAGAGGCACCAGC-3'	SEQ ID NO:21
5' -GAGAGAGGATCCGACCTAGGCGCGGAGGTACCGGCGCGACGGCG-3'	SEQ ID NO:22
5' -GAGAGACCTAGGAAGCCGGTGTTCGTGTTCCCGGCCAGGGCT-3'	SEQ ID NO:23
5' -GAGAGAGGATCCGAGGCCGGCCGTGCGCCCGGACCGAAGACCGCCTC-3'	SEQ ID NO:24
5' -GAGAGAATTCCTTAGGGTCGCCTTCGTCTTTCCCGGGCAGG-3'	SEQ ID NO:25
5' -TTGAGATCTTATGCATACGAGGGAAGCGGCACCTGTC-3'	SEQ ID NO:26

Mass spectrometry was routinely performed with a Finnigan-MAT 7000 mass spectrometer equipped with an atmospheric pressure chemical ionization source (APCI). Electrospray mass spectrometry (ESI-MS) was performed with a Finnigan-MAT 752-7000 mass spectrometer equipped with a Finnigan atmospheric pressure ionization (API) source. HPLC separation was carried out on a Hewlett-Packard 1050 liquid chromatograph using a Prodigy ODS (2) column (5µm, 50x2mm) and a gradient elution of 5mM ammonium acetate and methanol. The flow rate was 0.3 mL/min.

10

For large scale preparation of erythromycin derivatives, fermentation beers are typically adjusted to pH 9 with  $\text{NH}_4\text{OH}$  and then extracted two times with an equal volume of  $\text{CH}_2\text{Cl}_2$ . The pooled extract is then concentrated to a wet oil (approx. 1 g per liter of fermentation beer). Concentrated extracts are digested in methanol and chromatographed over a column of Sephadex® LH-20 (Pharmacia Biotech, Uppsala, Sweden) in the same solvent. Fractions are tested for bioactivity against *Staphylococcus aureus*, and active fractions are combined and concentrated. When additional column chromatography is desired to reduce sample weight, the concentrated sample is digested in a solvent system consisting of n-heptane, chloroform, ethanol (10:10:1, v/v/v) and chromatographed over a column of Sephadex® LH-20 in the same system. Fractions are then analyzed by  $^1\text{H}$  NMR, focusing on the characteristic erythromycin resonances around  $\delta = 5.0$  (H-13),  $\delta = 4.9$  (H-1''), and  $\delta = 4.4$  (H-1') (Everett and Tyler, J. Chem. Soc. Perkin Trans. I, pg. 2599 (1985)) and pooled according to purity. Alternatively, column chromatography is replaced with an extraction sequence. In this case, the initial pooled  $\text{CH}_2\text{Cl}_2$  extract is concentrated to approximately 400 mL. This is extracted twice with equal volumes of 0.05 M aqueous potassium phosphate with the pH chosen between pH 4.5-6. The aqueous phase is then pooled, adjusted to pH 8-9, and extracted twice with equal volumes of ethyl acetate. Finally, the ethyl acetate extracts are pooled and concentrated. When additional reduction in sample weight is desired, the extraction sequence is repeated on a 10-50 fold smaller scale, typically yielding about 500 mgs of partially pure material.

High resolution separation of erythromycin derivatives is obtained by one or more rounds of countercurrent chromatography (Hostettmann and Marston, *Anal. Chim. Acta*, 236:63-76 (1990)). When the weight of the partially pure sample from column chromatography or the extraction sequence is less than 5 g, but greater than 0.5 g, it is digested in 7 mL of the upper phase of a solvent system (3:7:5, v/v/v) consisting of n-hexane, ethyl acetate, 0.02 M aqueous potassium phosphate, with a pH chosen between 6.5-8.0, and chromatographed on a custom droplet countercurrent chromatography (DCCC) instrument [100 vertical columns, 0.4 cm dia. x 24 cm length; Hostettmann and Marston, *Anal. Chim. Acta*, 236:63-76 (1990)] in the same system with the upper phase as the mobile phase. Flow rates of approximately 120-200 mL/hr are employed. As before, fractions are analyzed by NMR and bioactivity, and pooled according to purity. When the weight of the partially pure sample is approximately 0.5 g or less, countercurrent chromatography is carried out on an Ito multi-layered horizontal Coil Planet Centrifuge (P.C. Inc., Potomac, MD) using either the system consisting of n-hexane, ethyl acetate, 0.02 M aqueous potassium phosphate, with the pH chosen between 6.5-8.0, (3:7:5, v/v/v) employed above, or similar systems in which the ratio of hexane to EtOAc and/or the pH are varied. The chromatography is developed either isocratically, or with a gradient starting, for example, with the upper phase of a solvent system consisting of n-hexane, ethyl acetate, 0.02 M aqueous potassium phosphate, with the

pH chosen between 6.5-8.0, (7:3:5, v/v/v) and finishing with the upper phase of a solvent system consisting of n-hexane, ethyl acetate, 0.02 M aqueous potassium phosphate, at the same pH, (1:1:1, v/v/v). In all cases, flow rates of approximately 120 mL/hr are employed. As before, fractions are analyzed by NMR and bioactivity, and pooled according to purity.

Once sufficient purity is achieved,  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra are measured with a General Electric GN500 spectrometer and structural assignments are made with the aid of with the aid of correlational spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond correlation (HMBC), and distortionless enhancement by polarization transfer (DEPT) experiments.

The foregoing can be better understood by reference to the following examples, which are provided as non-limiting illustrations of the practice of the instant invention.

EXAMPLE 1: Cloning of the LigAT2 Domain from  
*Streptomyces hygroscopicus* ATCC 29253

A genomic library of *Streptomyces hygroscopicus* ATCC 29253 DNA was constructed in the bifunctional cosmid pNJ1 (Tuan, *et al.*, *Gene* **90**: 21-29 (1990)) using standard methods of recombinant DNA technology. Briefly, cosmid vector was prepared by digesting approximately 5  $\mu\text{g}$  of pNJ1 with *Eco*RI, dephosphorylating with calf intestinal alkaline phosphatase (CIAP) and then digesting with *Bgl*II to generate one arm and also digesting 5  $\mu\text{g}$  of pNJ1 with *Hind*III, dephosphorylating with CIAP and then digesting with *Bgl*II to generate the other. Insert DNA was prepared by partially digesting approximately 25  $\mu\text{g}$  of high molecular weight *S. hygroscopicus* chromosomal DNA with *Sau*IIIa according to the procedure outlined in Maniatis, *et al. supra*. *Sau*IIIa fragments of approximately 35 kb were recovered from a 0.5% low melting point agarose gel by melting the appropriate gel slice to 65°C, adding 3 volumes of TE buffer, gently extracting 2X with phenol and once with chloroform and ethanol precipitating the aqueous phase. For the ligation, approximately 3  $\mu\text{g}$  of this chromosomal DNA was mixed with approximately 0.5  $\mu\text{g}$  of each cosmid arm and EtOH precipitated. The precipitate was resuspended in 7  $\mu\text{L}$  of water to which was added 2  $\mu\text{L}$  of 5X ligation buffer and 1  $\mu\text{L}$  of T4 DNA ligase. The mixture was incubated overnight at 16°C. GigapackII XL (Stratagene®) was used for packaging 2  $\mu\text{L}$  of the ligation mix according to the manufacture's instructions. The host bacterium was *E. coli* ER1772 from New England Biolabs (Beverly, MA). Twenty-six colonies were examined by restriction analysis and all were found to contain insert DNA. Individual colonies were picked into thirty-four 96-well plates to give a 99.99% probability that the library represented all *S. hygroscopicus* sequences. Further restriction analysis demonstrated the average insert size to be about 30 kb.

The library was screened with a 1.45 kb *Sst*I-*Msc*I DNA fragment encompassing the ketosynthase (KS) domain from module 5 of the erythromycin PKS gene *eryAIII* (Donadio

and Katz, 1992, *Gene*, **111**: 51-60). The DNA fragment was labeled with  $^{32}\text{P}$  using the Megaprime DNA labeling system (Amersham Life Science, Arlington Heights, IL). Colonies (3600) were transferred from 96-well plates to Hybond-N nylon membranes (Amersham Life Science, Arlington Heights, IL) and probed according to procedures outlined in Maniatis, *et al. supra*. Hybridization was performed at 65°C and a stringency wash carried out with 0.1x SSC at 65°C. About 60 cosmid clones were chosen which gave the strongest signals with this PKS probe.

We also decided to screen Southern digests of these clones with a second probe in order to identify potential genetically linked peptide synthetases in this strain. The probe was designed from conserved motifs of nonribosomal peptide synthetases (Borchert *et al.*, 1992, *FEMS Microbiology Letters*, **92**: 175-180) and consisted of a mixture of two degenerative 35-mers, SEQ ID NO:3 and SEQ ID NO:4. The mixed probe was labeled using DNA 5' End Labeling System (Promega Corp., Madison, WI). The 60 cosmid clones were digested with *Sma*I and run on 0.9% agarose gels. Southern analysis was performed according to Maniatis, *et al. supra*, except that hybridization was overnight at 55°C and the stringency wash was with 0.5x SSC at 55°C. Two cosmids, 54 and 58, were identified using this second probe. Thirteen additional cosmids were subsequently isolated by re-probing the cosmid library with a 1kb fragment from the left of the insert of cosmid 58. Two of these thirteen cosmids, designated A15 and A16, were then further analyzed by restriction analysis and DNA sequencing. Restriction and sequence analysis of a 32.8 kb continuous segment of DNA from A16 revealed a type I PKS cluster with four PKS modules. A genetic map of the cluster is shown in FIG. 6. Since an unusual CoA ligase-like domain was found in ORF1 (PKS1), the cluster was named "Lig-PKS".

The nucleotide sequence of the LigAT2 domain from Lig-PKS (top strand) and its corresponding amino acid sequence (bottom strand) are shown in FIG. 7 (SEQ ID NO:1 and SEQ ID NO:31 respectively). When SEQ ID NO:31 was compared with the 14 AT domains in the rapamycin PKS (Growtree Program, GCG, Madison WI), it was found to cluster with malonate-specifying rapamycin domains (see Growtree analysis of FIG. 3). Therefore, it was predicted that the LigAT2 specifies malonate as its cognate extender unit during synthesis of the polyketide encoded by Lig-PKS.

#### EXAMPLE 2: Construction of plasmid pUC18/LigAT2

Two PCR oligonucleotides (SEQ ID NO:5 and SEQ ID NO:6) were designed to subclone the 985 bp DNA segment encoding the LigAT2 domain from the Lig-PKS cluster and to introduce two unique restriction sites, *Avr*II and *Nsi*I, for cassette cloning. The unique restriction sites *Avr*II and *Nsi*I required for cassette cloning of the AT-encoding DNA were chosen based on multiple sequence alignment using the programs PILEUP and PRETTY (GCG, Madison WI) which compared the amino acid sequences of LigAT2, venAT, rapAT2,

rapAT5, rapAT8, rapAT9, rapAT11, rapAT12, rapAT14, eryAT1, eryAT2, eryAT3, eryAT4, eryAT5, eryAT6, and a monofunctional AT from *Streptomyces glaucescens* (R.G. Summers *et al.*, *Biochemistry* **34**:9389-9402 (1995)). The selection and positioning of the restriction enzyme sites were based on the following considerations: (i) extent of amino acid sequence conservation among the various ATs, with the sites being positioned outside, but near the regions of greatest conservation, (ii) absence of the sites from the heterologous AT-encoding DNA and the eryAT flanking DNA and (iii) impact of the amino acid sequence changes resulting from translation of these sites on the heterologous AT amino acid sequence. This necessitated nucleotide changes, shown in bold in FIG. 8, at the beginning and near the end of the LigAT2-encoding DNA sequence. (In FIG. 8, the underlined nucleotides are the wild-type sequence.) In addition, two other restriction sites, *Eco*RI and *Bam*HI, were also introduced at the 5' ends of the N-terminal and C-terminal oligonucleotides, respectively, for convenient subcloning of the PCR-generated product. The approximately 1 kb LigAT2 domain was amplified from Cosmid 58 as follows: The 100  $\mu$ L PCR reaction mixture contained 10  $\mu$ L of 10x PCR buffer (Bethesda Research Laboratories), 2  $\mu$ L of 10 mM dNTP mixture, 2-4  $\mu$ L of 50 mM MgCl<sub>2</sub>, 100 pM of each oligo, 10-50 ng of template DNA and water to 100  $\mu$ L. Cycling conditions were as follows: One cycle at 96°C/6 min, 80°C/1 min (add 5 U Taq DNA Polymerase during this 1 min) and 72°C/2 min; 30 cycles at 95°C/1 min, 65°C/1 min and 72°C/2 min with a 5 min extension at 72°C for the last cycle. The entire reaction was then run on a 1% agarose gel and the desired fragment was isolated with Prep-A-Gene (BioRad, Hercules, CA). The PCR product was digested with *Eco*RI and *Bam*HI and subcloned into the *Eco*RI and *Bam*HI sites of pUC18. The ligation mixture was transformed into *E. coli* DH5 $\alpha$  (GIBCO BRL) according to the manufacturer's instructions and transformants were selected on LB plates containing 150  $\mu$ g/mL ampicillin and 50  $\mu$ L of a 2% solution of X-gal for blue/white selection. Clones were confirmed by restriction analysis and the fidelity of the insert was confirmed by DNA sequencing. The final plasmid construct was named pUC18/LigAT2.

### EXAMPLE 3: Construction of plasmid pEryAT1/LigAT2

pEryAT1/LigAT2 was constructed using standard methods of recombinant DNA technology according to the schematic outlines of FIGS. 9 and 10. To construct a gene-replacement vector specific for the eryAT1 domain, the two DNA regions immediately adjacent to eryAT1-encoding DNA were cloned and positioned adjacent to the LigAT2-encoding DNA as described in Example 2. The 5' and 3' boundaries of eryAT1 were designated as 3825 and 4866, and correspond to the deposited *eryA1* sequence (GenBank accession number M63676). To subclone the DNA fragment upstream of the eryAT1 domain encoding region from the *Sac. erythraea* chromosome, two PCR oligonucleotides (SEQ ID NO:7 and SEQ ID NO:8) were designed so that an *Eco*RI site was added at the 5' end of the



region and *AvrII*-*Bam*HI restriction sites were introduced at the 3' end. The 5'-flanking region (about 1 kb) was PCR generated as described in EXAMPLE 2 using plasmid pAIEN22 DNA as template. (This plasmid is a pUC19 derivative containing 22 kb of *Sac. erythraea* DNA from an *Eco*RI site upstream of *eryA*I to an *Nhe*I site in *eryA*II cloned into *Eco*RI and *Xba*I cut pUC19). The PCR product was subcloned into *Eco*RI and *Bam*HI sites of pUC19 and the ligated DNA transformed into *E. coli* DH5 $\alpha$  (GIBCO BRL) according to the manufacturer's instructions. Clones were selected on LB plates containing 150  $\mu$ g/ml. ampicillin and 50  $\mu$ L of a 2% solution of X-gal for blue/white selection. Clones were confirmed by restriction analysis and the fidelity of the insert was confirmed by DNA sequencing. The resulting construct was named pUC19/AT1/5'-flank.

For subcloning the 3'-flanking region of the *eryA*T1 from *Sac. erythraea* chromosome, two PCR oligonucleotides (SEQ ID NO:9 and SEQ ID NO:10) were designed so that *Bam*HI-*Nsi*I restriction sites were introduced into the 5' end of the region and a *Hind*III restriction site was added to the 3' end. The 3'-flanking region (about 1 kb) was also generated by PCR using pAIEN22 as template as described above. The PCR fragment was subcloned into the *Bam*HI and *Hind*III sites of pUC19 and the ligated DNA transformed into *E. coli* DH5 $\alpha$  as above. Clones were selected on LB plates containing 150  $\mu$ g/ml. ampicillin and 50  $\mu$ L of a 2% solution of X-gal for blue/white selection. Clones were confirmed by restriction analysis and the fidelity of the insert was confirmed by DNA sequencing. This intermediate construct was named pUC19/AT1/3'-flank. The two flanking regions were joined by first isolating the 1 kb *Bam*HI-*Hind*III fragment (3'-flank) from pUC19/AT1/3'-flank and then ligating this fragment to pUC19/AT1/5'-flank cut with *Bam*HI and *Hind*III. Ligated DNA was transformed into *E. coli* DH5 $\alpha$  and clones isolated as described. The resulting plasmid was named pUC19/AT1-flank. The 2.1 kb *Eco*RI and *Hind*III fragment from pUC19/AT1-flank was then isolated and ligated to pCS5 cut with the same enzymes to generate pCS5/AT1-flank. The final step in the construction of pEryAT1/LigAT2 was to ligate the 1 kb LigAT2 fragment having *Avr*II and *Nsi*I ends to pCS5/AT1-flank cut with the same enzymes to give the gene replacement/integration plasmid pEryAT1/LigAT2. All ligation mixtures were transformed into the intermediate host *E. coli* DH5 $\alpha$  and clones selected as previously described.

#### EXAMPLE 4: Construction of *Sac. erythraea* ER720 EryAT1/LigAT2

An example of a 12-desmethyl-12-deoxyerythromycin A producing microorganism was prepared by replacing the DNA fragment encoding the methylmalonyl acyltransferase domain in module 1 of the erythromycin PKS (EryAT1) of *Sac. erythraea* ER720 with a newly discovered DNA fragment encoding a malonyl acyltransferase domain (LigAT2) from *S. hygroscopicus* ATCC 29253. This was accomplished with the recombinant plasmid, pEryAT1/LigAT2, prepared as described in Example 3. Transformation of *Sac. erythraea*

ER720 and resolution of the integration event were carried out according to the following method. *Sac. erythraea* ER720 cells were grown in 50 mL of SGGP medium (per 1 liter aqueous solution: 4 g peptone, 4 g yeast extract, 4 g casamino acids, 2 g glycine, 0.5 g  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ , 10 g glucose, 20 mL of 500 mM  $\text{KH}_2\text{PO}_4$ ) for 3 days at 32 °C and then washed in 10 mL of 10.3% sucrose. The cells were resuspended in 10 mL of P<sub>M</sub> buffer containing 1 mg/mL lysozyme and incubated at 30 °C for 15-30 minutes until most of the mycelial segments were converted into spherical protoplasts. (P<sub>M</sub> buffer per 1 liter aqueous solution: 200 g sucrose, 0.25 g  $\text{K}_2\text{SO}_4$  in 890 mL  $\text{H}_2\text{O}$ , with the addition after sterilization of 100 mL 0.25 M TES, pH 7.2, 2 mL trace elements solution (Hopwood, *et al.*, 1985, Genetic Manipulation of Streptomyces A Laboratory Manual, The John Innes Foundation), 0.08 mL 2.5 M  $\text{CaCl}_2$ , 10 mL 0.5%  $\text{KH}_2\text{PO}_4$ , 2 mL 2.5 M  $\text{MgCl}_2$ .) The protoplasts were washed once with P<sub>M</sub> and then resuspended in 3 mL of the same buffer containing 10% DMSO for storage in 200 µL aliquots at -80 °C.

Transformation was accomplished by quickly thawing an aliquot of protoplasts, centrifuging for 15 seconds in a microfuge, decanting the supernatant, and resuspending the protoplasts in the P<sub>M</sub> remaining in the tube. Ten µL of DNA solution was added (3 µL of pEryAT1/LigAT2 DNA from Example 3 at about 1 µg/µL in 7 µL of P<sub>M</sub> buffer) and mixed with the protoplasts by gently tapping the tube. Two tenths of a mL of 25% PEG 8000 in T buffer (Hopwood, *et al.*, 1985, Genetic Manipulation of Streptomyces A Laboratory Manual, The John Innes Institute) was then added, mixed by pipetting the solution 3 times and the suspension immediately spread on a dried R3M plate. The plate was incubated at 30 °C for 20 hours and overlaid with 2 mL of water containing 100 µg/mL thiostrepton, dried briefly and incubated 4 more days at 30 °C.

To select stable transformants (integrants) colonies arising on the transformation plates were re-streaked onto R3M plates containing thiostrepton (20 µg/mL). Two colonies were confirmed to be thiostrepton resistant and one of these was inoculated into SGGP containing thiostrepton (10 µg/mL) to isolate chromosomal DNA for Southern analysis. Integration of the plasmid DNA into the ER720 chromosome was further confirmed by Southern hybridization (data not shown). Hybridization was at 65°C and the stringency wash was with 0.1x SSC at 65°C.

The confirmed integrant was grown in SGGP without antibiotic for four days and then plated onto non-selective R3M plates for sporulation. Spores were plated on R3M plates to obtain individual colonies, which were then screened for sensitivity to thiostrepton, indicating loss of the plasmid sequence from the chromosome. Five thiostrepton sensitive colonies were selected and 3 of these were confirmed by Southern hybridization to have the EryAT1 replaced by LigAT2 (FIG. 11). Hybridization was at 65°C and the stringency wash was with 0.1x SSC at 65°C. The strain was named *Sac. erythraea* ER720 EryAT1/LigAT2.

EXAMPLE 5: Analysis of compounds produced by *Sac. erythraea* ER720 EryAT1/LigAT2

Compounds produced by the recombinant *Sac. erythraea* strain, ER720

EryAT1/LigAT2, whose construction is described in Example 4, were characterized by TLC, bioautography, mass spectrometry and NMR analysis.

For TLC analysis cells were grown in either SGGP or SCM medium (20 g Soytone, 15 g Soluble Starch, 10.5 g MOPS, 1.5 g Yeast Extract and 0.1 g  $\text{CaCl}_2$  per liter of distilled  $\text{H}_2\text{O}$ ) for 4-5 days at  $30^\circ\text{C}$ . 1.5 mL of culture was centrifuged for 1 minute in a microfuge to remove cells. One mL of the resulting supernatant was removed to another microfuge tube and the pH adjusted to 9.0 by the addition of 6  $\mu\text{L}$  of  $\text{NH}_4\text{OH}$ . Then 0.5 mL of ethyl acetate was added, the tube was vortexed for 10 sec and then centrifuged for approximately 5 min to achieve phase separation. The organic phase was removed to another tube, and the aqueous phase was re-extracted with 0.5 mL of ethyl acetate. The second organic phase was combined with the first and dried in a Speed Vac. The residue was taken up in 10  $\mu\text{L}$  of ethyl acetate and 5  $\mu\text{L}$  was spotted onto a Merck 60F-254 silica gel TLC plate. The plate was run in isopropyl ether:methanol: $\text{NH}_4\text{OH}$  (75:35:2). Erythromycin derivatives were visualized by spraying the plates with anisaldehyde:sulfuric acid:ethanol (1:1:9). Using this reagent, a novel compound predicted to be 12-desmethyl-12-deoxyerythromycin A, appeared as a blue spot running slightly faster than erythromycin A (FIG. 12).

To detect biological activity, a TLC-bioautography assay was performed. In this assay, one microliter of the extracted sample from above was spotted onto a TLC plate which was run as described above. The plate was then air-dried and placed in a sterile bio-assay dish (245x245x25 mm). The plate was then covered with 100 mL of antibiotic medium 11 (DIFCO-BACTO) containing *Staphylococcus aureus* as an indicator strain and incubated overnight at  $37^\circ\text{C}$ . As with the positive controls, a clear zone of inhibition developed around the sample spot indicating that the novel compound had bioactivity.

To determine whether the novel spot seen on TLC had the molecular mass corresponding to the predicted 12-desmethyl-12-deoxyerythromycin A, an ethyl acetate extract was further analyzed by mass spectrometry. The mass spectrometry samples were isolated by TLC basically as described above except that plates were not sprayed with the anisaldehyde reagent. The region of the novel spot was instead scraped from the TLC plate and the silica resin re-extracted with ethyl acetate-methanol (1:1) and then twice with ethyl acetate. The combined solvent phases were then dried in a Speed Vac. Mass spectrometric analysis revealed the novel compound to have a mass of 704, which corresponds to the molecular ion plus a proton ( $\text{M}+\text{H}^+$ ) of 12-desmethyl-12-deoxyerythromycin A.

To acquire milligram quantities of highly purified material for performance of NMR analysis, the culture was grown in a 42-liter LH Fermentation Series 2000 fermentor. SCM medium was used for growth of inoculum and for the fermentation. Seed for the fermentation was grown in two steps. In the first step, frozen vegetative inoculum was used

to seed 100 mL of SCM medium in a 500 mL Erlenmeyer flask. For the second step, 2-liter Erlenmeyer flasks containing 600 mL of SCM medium were seeded at 5% from the first passage growth. Each step was incubated for 3 days at 32 °C on a rotary shaker operated at 225 rpm.

Thirty liters of SCM medium were prepared in the 42-liter fermentor and sterilized at 121°C and 15 psi for 1 hour. Antifoam (XFO-371, Ivanhoe Chemical Co., Mundelein, IL) was added initially at 0.01% and then was available on demand. The fermentor was inoculated with 1.5 liters of the second passage seed growth. The temperature was controlled at 32°C. The agitation rate was 260 rpm and the air flow was 1.3 vol/vol/min. The head pressure was maintained at 6 psi. During fermentation pH was controlled at 7.3 with 5 M propionic acid. The fermentation was terminated at 111 hours, and the fermentation beer was adjusted to pH 8. This was followed by two extractions with equal volumes of CH<sub>2</sub>Cl<sub>2</sub>. The pooled CH<sub>2</sub>Cl<sub>2</sub> extract was then concentrated to approximately 400 mL and extracted twice with equal volumes of 0.05 M aqueous potassium phosphate pH 5.5. The aqueous phase was pooled and adjusted to pH 8, and then extracted twice with equal volumes of ethyl acetate. The ethyl acetate extracts were pooled and concentrated to yield 5 ml oil. The extraction sequence described above was then repeated to yield 600 mg of oil after concentration. Next, the sample was split and each half was digested in 2.5 ml each of the upper and lower phases of a solvent system consisting of n-hexane, ethyl acetate, 0.02 M aqueous potassium phosphate, pH 8, (1:1:1, v/v/v). These were then chromatographed on the Coil Planet Centrifuge using the upper phase as the mobile phase. Fractions were analyzed by bioassay against *Staphylococcus aureus* and <sup>1</sup>H NMR. Two macrolide containing peaks of bioactivity were observed in both samples, and the later eluting peaks from each sample, which contained most of the bioactivity, were pooled and concentrated. The concentrated material was then digested in 2.5 mL each of the upper and lower phases of a solvent system consisting of n-hexane, ethyl acetate, 0.02 M aqueous potassium phosphate, pH 6.5, (6:4:5, v/v/v), and was chromatographed on the Coil Planet Centrifuge using the upper phase as the mobile phase. Fractions were analyzed by bioassay and <sup>1</sup>H NMR. Two macrolide containing peaks of bioactivity were observed and the later eluting species was readily characterized by its <sup>1</sup>H and <sup>13</sup>C NMR spectra as 12-desmethyl-12-deoxyerythromycin A. Parameters from the <sup>1</sup>H NMR spectra are listed in Table 2. The assignments were made with the aid of correlational spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond correlation (HMBC), and distortionless enhancement by polarization transfer (DEPT) experiments. Mass spectral data of this sample was also consistent with the structural assignment. Electrospray ionization (ESI) of this sample revealed an M+H<sup>+</sup> ion at M/Z 704, which is in full accord with erythromycin A lacking both a methyl group and a hydroxyl group.

Table 2  
<sup>1</sup>H NMR chemical shift ( $\delta$ ) assignments for 12-desmethyl-12-deoxyerythromycin A  
in CDCl<sub>3</sub>

5	2-H	2.74	1'-H	4.47
	3-H	4.15	2'-H	3.25
	4-H	2.01	3'-H	2.49
	5-H	3.58	4'-H <sub>a</sub>	1.67
	7-H <sub>a</sub>	1.91	4'-H <sub>b</sub>	1.23
10	7-H <sub>b</sub>	1.66	5'-H	3.54
	8-H	2.86	6'-H <sub>3</sub>	1.23
	10-H	2.70	N(CH <sub>3</sub> ) <sub>2</sub>	2.30
	11-H	4.05	1''-H	4.85
	12-H <sub>a</sub>	1.71	2''-H <sub>a</sub>	2.40
15	12-H <sub>b</sub>	1.46	2''-H <sub>b</sub>	1.59
	13-H	5.06	4''-H	3.03
	14-H <sub>2</sub>	1.59	5''-H	4.04
	15-H <sub>3</sub>	0.89	6''-H <sub>3</sub>	1.30
	2-CH <sub>3</sub>	1.19	3''-CH <sub>3</sub>	1.25
20	4-CH <sub>3</sub>	1.13	OCH <sub>3</sub>	3.33
	6-CH <sub>3</sub>	1.38		
	8-CH <sub>3</sub>	1.19		
	10-CH <sub>3</sub>	1.11		

#### EXAMPLE 6: Construction of plasmid pEryAT2/LigAT2

pEryAT2/LigAT2 was constructed using standard methods of recombinant DNA technology. To make a gene-replacement vector specific for the eryAT2 domain, two DNA regions flanking eryAT2 were cloned and positioned adjacent to the DNA encoding the domain to be inserted in order to effect homologous recombination. Boundaries of the AT2 domain were chosen as described in Example 2. The 5' and 3' boundaries of eryAT2 are designated as 8255 and 9282, respectively, and correspond to deposited *eryAI* sequence (GenBank accession number M63676). To subclone the DNA fragment upstream of the eryAT2 DNA, two PCR oligonucleotides (SEQ ID NO:11 and SEQ ID NO:12) were designed so that a *Hind*III site was added at the 5' end of the region and *Avr*II-*Pst*I restriction sites were introduced at the 3' end. For subcloning the 3'-flanking region of eryAT2, two PCR oligonucleotides (SEQ ID NO: 13 and SEQ ID NO:14) were designed so that *Pst*I-*Nsi*I restriction sites were introduced at the 5' end of the region and an *Eco*RI site at the 3' end. Both the 5'-flanking and 3'-flanking regions (about 1 kb each) were PCR generated as described in Example 3. In the case of the 5'-flanking region, the PCR product was subsequently subcloned into *Hind*III and *Pst*I sites of pUC18 whereas the PCR product of the 3'-flanking region was subcloned into the *Pst*I and *Eco*RI sites of pUC18. Ligations, transformations and confirmations of selected clones were performed as in Example 3. The resulting construct containing the AT2 5'-flanking region was designated pUC18/AT2/5'-

flank and the construction containing the AT2 3'-flanking region was designated pUC18/AT2/3'-flank. The two flanking regions were then joined by first isolating the 1 kb *Pst*I and *Eco*RI fragment (3'-flank) from pUC18/AT2/3'-flank, and ligating this fragment to pUC18/AT2/5'-flank cut with *Pst*I and *Eco*RI. The ligation was transformed into *E. coli* DH5 $\alpha$  and clones isolated as described. The resulting plasmid was named pUC18/AT2-flank (FIG. 13). The 2.2 kb *Eco*RI and *Hind*III fragment from pUC18/AT2-flank was then isolated and ligated to pCS5 cut with the same enzymes to generate pCS5/AT2-flank. The final step in the construction of pEryAT2/LigAT2 was to ligate the LigAT2 encoding DNA fragment from pUC18/LigAT2 having *Avr*II and *Nsi*I ends (described in Example 2) to pCS5/AT2-flank cut with the same enzymes to give the gene replacement, integration plasmid pEryAT2/LigAT2 (FIG. 14). All ligations were transformed into the intermediate host *E. coli* DH5 $\alpha$  and clones selected as previously described.

#### EXAMPLE 7: Construction of *Sac. erythraea* ER720 EryAT2/LigAT2

An example of a 10-desmethylerythromycin A and 10-desmethyl-12-deoxyerythromycin A producing microorganism was prepared by replacing the methylmalonyl acyltransferase domain of module 2 of the erythromycin PKS (EryAT2) of *Sac. erythraea* ER720 with a newly discovered malonyl acyltransferase domain (LigAT2) from *S. hygroscopicus* ATCC 29253. This was accomplished with the recombinant plasmid, pEryAT2/LigAT2, prepared as described in Example 6. Transformation of ER720 and resolution of the integration event were carried out according to the procedures described in Example 4 using 10  $\mu$ L of a DNA solution consisting of 3  $\mu$ L of pEryAT2/LigAT2 DNA from Example 6 at about 1  $\mu$ g/ $\mu$ L in 7  $\mu$ L of PM buffer. Three colonies were confirmed to be thiostrepton resistant and were inoculated into SGGP containing thiostrepton (10  $\mu$ g/mL) to isolate chromosomal DNA for Southern analysis. Integration of the plasmid DNA into ER720 chromosome was further confirmed by Southern hybridization (data not shown). Hybridization was at 65°C and the stringency wash was with 0.1x SSC at 65°C.

The confirmed integrant was grown in SGGP without antibiotic for four days and then plated onto non-selective R3M plates for sporulation. Spores were plated on R3M plates to obtain individual colonies, which were then screened for sensitivity to thiostrepton, indicating loss of the plasmid sequence from the chromosome. Two thiostrepton sensitive colonies were selected and one of these was confirmed by Southern hybridization to have the EryAT2 replaced by LigAT2 (FIG. 15). Hybridization was at 65°C and the stringency wash was with 0.1x SSC at 65°C. The strain was named *Sac. erythraea* ER720 EryAT2/LigAT2.

#### EXAMPLE 8: Analysis of compounds produced by *Sac. erythraea* ER720 EryAT2/LigAT2

Compounds produced by the recombinant *Sac. erythraea* strain, ER720 EryAT2/LigAT2, whose construction is described in Example 7, were characterized by TLC, bioautography and mass spectrometry.

For small scale analysis, the cells were grown in either SGGP or SCM medium for 4-5 days at 30°C. 15 mL of culture was centrifuged for 10 minute in a Sorval GLC-4 General Laboratory Centrifuge at setting 10 to remove cells. Ten mL of the resulting supernatant was pH adjusted to 9.0 by the addition of 60 µL of NH<sub>4</sub>OH. Then 5 mL of ethyl acetate was added, the tube was shaken vigorously for 3 minutes and then centrifuged for approximately 5 min to achieve phase separation. The organic phase was removed to another tube, and the aqueous phase was re-extracted with 5 mL of ethyl acetate. The second organic phase was combined with the first and dried in a Speed Vac. The residue was taken up in 20 µL of ethyl acetate and 10 µL was spotted onto a Merck 60F-254 silica gel TLC plate. The plate was run in isopropyl ether:methanol:NH<sub>4</sub>OH (75:35:2). Erythromycin derivatives were visualized by spraying the plates with anisaldehyde:sulfuric acid:ethanol (1:1:9). Using this reagent, two novel compounds predicted to be 10-desmethylethromycin A and 10-desmethyl-12-deoxyerythromycin A, appeared as blue spots with the lower spot running slightly slower than erythromycin A and upper spot running slightly faster than erythromycin A (FIG. 16).

To detect biological activity, a TLC-bioautography assay was performed. In this assay, 0.2 to 1 microliter of the extracted sample from above was spotted onto a TLC plate which was run as described. The plate was then air-dried and placed in a sterile bio-assay dish (245x245x25 mm). The plate was then covered with 100 mL of antibiotic medium 11 (DIFCO-BACTO) containing *Staphylococcus aureus* as an indicator strain. The inhibition zones were developed by overnight incubation of the plate at 37 °C. As shown in FIG. 17 (TLC-bioautography), the two novel spots (compounds) each have bioactivity against *Staphylococcus aureus*.

To determine whether the novel spots seen on TLC had the molecular masses corresponding to the predicted 10-desmethylethromycin A and 10-desmethyl-12-deoxyerythromycin A, an ethyl acetate extract was further analyzed by mass spectrometry. The mass spectrometry samples were isolated by TLC similarly to the method described above except that plates were not sprayed with the anisaldehyde reagent. Instead, two regions which contain the novel spots were scraped from the TLC plate and the silica resin re-extracted with ethyl acetate-methanol (1:1) and then twice with ethyl acetate. The combined solvent phases were then dried in a Speed Vac. In addition to the samples described above, a crude ethyl acetate extract was also analyzed by LC-MS, in which the sample components were first separated by liquid chromatography and then analyzed by mass spectrometry. Mass spectrometric analysis revealed the two novel compounds to have masses of 720 and 704, which correspond to the molecular ion plus a proton (M+H<sup>+</sup>) of 10-desmethylethromycin A and 10-desmethyl-12-deoxyerythromycin A, respectively.

### EXAMPLE 9: Cloning of the venAT Domain from *Streptomyces venezuelae*

A genomic library of *Streptomyces venezuelae* ATCC 15439 DNA was constructed in the bifunctional cosmid pNJ1 (Tuan, *et al.*, *Gene* **90**: 21-29 (1990)) using standard methods of recombinant DNA technology. A cosmid from this library, pVen17, was characterized by Southern analysis and *Sst*I fragments of approximately 3.5, 3.8, and 4.0 kb were found to hybridize to a 1.37 kb *Sma*I fragment that encompasses the ketosynthase (KS) domain from module 2 of the erythromycin PKS gene *eryA*I (Donadio *et al.*, *Science* **252**: 675-679 (1991)). The 4.0 kb *Sst*I fragment was then subcloned into pUC19 to give pVen4.0. The nucleotide sequence of pVen4.0 insert DNA was determined from single strand DNA templates prepared from M13mp18 and M13mp19 (Yanisch-Perron, *et al.*, *Gene*, **33**:103 (1985)) subclones using Sequenase version 2.0 with 7-deaza-dGTP (United States Biochemical, Cleveland, OH) and 5'-[ $\alpha$ -<sup>32</sup>P] or 5'-[ $\alpha$ -<sup>33</sup>P]-dCTP (NEN Research Products, Boston, MA). Because pVen4.0 did not contain the entire AT domain, the nucleotide sequence was extended using pVen17 DNA as the template. The nucleotide sequence of the venAT domain (SEQ ID NO:2) and its corresponding amino acid sequence (SEQ ID NO:32) is shown in FIG. 18 (top and bottom strands respectively).

### Example 10: Construction of plasmid pEryAT1/venAT

pEryAT1/venAT was constructed using standard methods of recombinant DNA technology according to the schematic outlines of FIGS. 23 and 24. Two PCR oligonucleotides (SEQ ID NO:15 and SEQ ID NO:16) were designed to subclone the 1.03 kb DNA fragment that encodes the venAT domain (FIG. 19) from the *S. venezuelae* PKS cluster and to introduce two unique restriction sites, *Avr*II and *Nsi*I, for cassette cloning (described in Example 2). This necessitated nucleotide changes (shown in bold in FIG. 19) at the beginning and near the end of the venAT sequence (underlined nucleotides are the wild-type sequence). In addition, two other restriction sites, *Eco*RI and *Bam*HI, were also introduced at the 5' ends of the N-terminal and C-terminal oligonucleotides, respectively, for convenient subcloning of the PCR-generated product. The approximately 1 kb venAT-encoding DNA was PCR amplified from cosmid pVen17 template DNA (Example 2) using VentR® DNA Polymerase (New England Biolabs). A typical PCR reaction contained 10  $\mu$ L ThermoPol Buffer, 10  $\mu$ L formamide, 10  $\mu$ L of 20% glycerol, 55  $\mu$ L water, 100 pmole of each primer,



digested pUC18 and transformed into *E. coli* DH5 $\alpha$  (GIBCO BRL) according to the manufacturer's instructions. Clones were selected on LB plates containing 150  $\mu$ g/mL ampicillin and 50  $\mu$ L of a 2% solution of X-gal for blue/white selection. Clones were confirmed by restriction analysis and the fidelity of the insert was confirmed by DNA sequencing. The final construct was named pUC18/venAT.

The final step in the construction of pEryAT1/venAT was to ligate the 1 kb venAT fragment having *Avr*II and *Nsi*I ends to pCS5/AT1-flank (Example 3) cut with the same enzymes to give the gene replacement/integration plasmid pEryAT1/venAT (FIG. 20). All ligations were transformed into the intermediate host *E. coli* DH5 $\alpha$  and clones selected as previously described.

#### Example 11: Construction of *Sac. erythraea* ER720 EryAT1/venAT

A 12-desmethyl-12-deoxyerythromycin A producing microorganism was prepared by replacing the methylmalonyl acyltransferase domain of module 1 of the erythromycin PKS (EryAT1) of *Sac. erythraea* ER720 with a newly discovered malonyl acyltransferase domain (venAT) from *S. venezuelae* ATCC 15439. This was accomplished with the recombinant plasmid, pEryAT1/venAT, prepared as in Example 10. Transformation of ER720 and resolution of the integration event were carried out as described in Example 4 using 10  $\mu$ L of DNA solution consisting of 3  $\mu$ L of pEryAT1/venAT DNA at about 1  $\mu$ g/mL in 7  $\mu$ L of PM buffer. One thiostrepton resistant colony was isolated and was inoculated into SGGP containing thiostrepton (10  $\mu$ g/mL) to isolate chromosomal DNA for Southern analysis. Integration of the plasmid DNA into the ER720 chromosome was further confirmed by Southern hybridization (data not shown). Hybridization was at 65°C and the stringency wash was with 0.1x SSC at 65°C.

The confirmed integrant was grown in SGGP without antibiotic for four days and then diluted 1000 fold into fresh medium and grown for 4 more days. Cells were then plated onto non-selective R3M plates for sporulation. Spores were plated on R3M plates to obtain individual colonies, which were then screened for sensitivity to thiostrepton, indicating loss of the plasmid sequence from the chromosome. Four thiostrepton sensitive colonies were selected and 2 of these were confirmed by Southern hybridization, using conditions described above, to have the EryAT1 replaced by venAT (FIG. 21). The strain was named *Sac. erythraea* ER720 EryAT1/venAT.

#### Example 12: Analysis of compounds produced by

##### *Sac. erythraea* ER720 EryAT1/venAT

Compounds produced by the recombinant *Sac. erythraea* strain, ER720 EryAT1/venAT, whose construction is described in Example 11, were characterized by TLC, bioautography, and mass spectrometry.

For TLC analysis cells were grown in either SGGP or SCM medium (20 g Soytone, 15 g Soluble Starch, 10.5 g MOPS, 1.5 g Yeast Extract and 0.1 g CaCl<sub>2</sub> per liter of distilled H<sub>2</sub>O) for 4-5 days at 30°C. The culture (1.5 mL) was centrifuged for 1 minute in a microfuge to remove cells. One mL of the resulting supernatant was removed to another microfuge tube and the pH adjusted to 9.0 by the addition of 6 µL of NH<sub>4</sub>OH. Then 0.5 mL of ethyl acetate was added, the tube was vortexed for 10 sec and then centrifuged for approximately 5 min to achieve phase separation. The organic phase was removed to another tube, and the aqueous phase was re-extracted with 0.5 mL of ethyl acetate. The second organic phase was combined with the first and dried in a Speed Vac. The residue was taken up in 10 µL of ethyl acetate and the entire sample was spotted onto a Merck 60F-254 silica gel TLC plate. The plate was run in isopropyl ether:methanol:NH<sub>4</sub>OH (75:35:2). Erythromycin derivatives were visualized by spraying the plates with anisaldehyde:sulfuric acid:ethanol (1:1:9). Using this reagent, a novel compound predicted to be 12-desmethyl-12-deoxyerythromycin A, appeared as a blue spot running slightly faster than erythromycin A (FIG. 22).

To detect biological activity, a TLC-bioautography assay was performed. In this assay, one µL of an extract prepared as above was spotted onto a TLC plate which was run as described above. The plate was then air-dried, placed face down on top of 100 mL of antibiotic medium 11 (DIFCO-BACTO) containing *Staphylococcus aureus* as an indicator strain in a sterile bio-assay dish (245x245x25 mm) and incubated overnight at 37°C. As with the positive controls, a clear zone of inhibition developed around the sample spot indicating that the novel compound was bioactive.

To determine whether the novel spot seen on TLC had the molecular mass corresponding to the predicted 12-desmethyl-12-deoxyerythromycin A, an ethyl acetate extract was further analyzed by mass spectrometry. The mass spec samples were isolated by TLC basically as described above except that plates were not sprayed with anisaldehyde. The region of the novel spot was instead scraped from the TLC plate and the silica resin re-extracted with ethyl acetate-methanol (2:1) and then twice with ethyl acetate. The combined solvent phases were then dried in a Speed Vac. Mass spectrometric analysis revealed the novel compound to have a mass of 704, which corresponds to the molecular ion plus a proton (M+H<sup>+</sup>) of 12-desmethyl-12-deoxyerythromycin A.

#### EXAMPLE 13: Construction of plasmid pUC19/rapAT14

Two PCR oligonucleotides (SEQ ID NO:17 and SEQ ID NO:18) were designed to subclone the 1023 bp rapAT14-encoding DNA fragment from the rapamycin biosynthetic gene cluster (GenBank Accession #: X86780) and to introduce two unique restriction sites, AvrII and NsiI, for cassette cloning (described in Example 2). This necessitated nucleotide changes (shown in bold in FIG. 23) at the beginning and near the end of the rapAT14 sequence. (In FIG. 23, the underlined nucleotides are the wild-type sequence.) In addition,

two other restriction sites, *EcoRI* and *HindIII*, were also introduced at the 5' ends of the N-terminal and C-terminal oligonucleotides, respectively, for convenient subcloning of the PCR-generated product. The approximately 1 kb rapAT14-encoding DNA was amplified by PCR using chromosomal DNA from *Streptomyces hygroscopicus* ATCC 29253 as template.

5 The PCR conditions were as follows: The 100  $\mu$ L reaction mixture contains 10  $\mu$ L of 10x Thermopol Buffer (New England Biolabs), 2% glycerol, 10% formamide, 100 pmoles of each oligo, 100-200 ng of template DNA and water to 84  $\mu$ L. The sample was then heated to 99° C for two minutes followed by cooling to 80° C for two minutes at which time 16  $\mu$ L of a dNTP solution (1.25 mM dATP and dTTP, 1.5 mM dCTP and dGTP) and 1  $\mu$ L of VentR®

10 DNA Polymerase (New England Biolabs) was added. Cycling was as follows: 30 cycles at 96.5° C/35 sec, 65° C/1 min and 72° C/1.5 min followed by one cycle at 72° C for 3 min. The entire reaction was then run on a 1.2% low-melting agarose gel and the desired fragment was isolated by melting the appropriate gel slice at 65° C, adding 3 volumes of TE buffer, extracting 2X with phenol and once with chloroform, and ethanol precipitating the aqueous

15 phase. The isolated DNA was ligated directly into *HincII* digested pUC19. The ligation mixture was transformed into *E. coli* DH5 $\alpha$  (GIBCO BRL) according to the manufacturer's instructions and transformants were selected on LB plates containing 150  $\mu$ g/mL ampicillin and 50  $\mu$ L of a 2% solution of X-gal for blue/white selection. Clones were confirmed by restriction analysis and the fidelity of the insert was confirmed by DNA sequencing. The

20 final plasmid construct was named pUC19/rapAT14.

#### EXAMPLE 14: Construction of plasmid pEryAT1/rapAT14

pEryAT1/rapAT14 was constructed using standard methods of recombinant DNA technology according to the schematic outlines of FIG. 24. To make a gene-replacement-

25 vector specific for the eryAT1 domain, the two DNA regions immediately adjacent to eryAT1 were cloned and positioned adjacent to the DNA encoding the rapAT14 domain in order to allow homologous recombination to occur. The strategy and protocol for constructing the intermediate plasmid containing the flanking regions, pCS5/AT1-flank, are described in Example 3 and FIG. 9. To insert the rapAT14 fragment between the flanking

30 regions, pUC19/rapAT14 (from Example 13) was digested with *NsiI* and *AvrII* and the resulting 1 kb fragment was isolated from a 0.8% agarose gel with Prep-A-Gene. pCS5/AT1-flank was also digested with these enzymes and the linearized plasmid was isolated from 0.8% agarose gel. The two fragments were ligated, transformed into the intermediate host *E. coli* DH5 $\alpha$  and ampicillin resistant clones were selected as previously described. Insertion of

35 the rapAT14 fragment between the ery flanking regions was confirmed by restriction analysis and the resulting plasmid was called pEryAT1/rapAT14.

#### EXAMPLE 15: Construction of *Sac. erythraea* ER720 EryAT1/rapAT14

An example of a 12-desmethyl-12-deoxyerythromycin A producing microorganism was prepared by replacing the methylmalonyl acyltransferase domain of module 1 of the erythromycin PKS (EryAT1) of *Sac. erythraea* ER720 with the acyltransferase domain from module 14 of the rapamycin PKS from *S. hygroscopicus* ATCC 29253. This was accomplished with the recombinant plasmid, pEryAT1/rapAT14, prepared as described in Example 14. Transformation of *Sac. erythraea* ER720 and resolution of the integration event were carried out according to the following method. *Sac. erythraea* ER720 cells were grown in 50 mL of SGGP medium (per 1 liter aqueous solution: 4 g peptone, 4 g yeast extract, 4 g casamino acids, 2 g glycine, 0.5 g  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ , 10 g glucose 20 mL of 500mM  $\text{KH}_2\text{PO}_4$ ) for 3 days at 32 °C and then washed in 10 mL of 10.3% sucrose. The cells were resuspended in 10 mL of PM buffer containing 1 mg/mL lysozyme and incubated at 30 °C for 15-30 minutes until most of the mycelial fragments were converted into spherical protoplasts. (PM buffer per 1 liter aqueous solution: 200 g sucrose, 0.25 g  $\text{K}_2\text{SO}_4$  in 890 mL  $\text{H}_2\text{O}$ , with the addition after sterilization of 100 mL 0.25 M TES, pH7.2, 2 mL trace elements solution (Hopwood, et al, 1985, Genetic Manipulation of Streptomyces A Laboratory Manual, The John Innes Foundation), 0.08 mL 2.5 M  $\text{CaCl}_2$ , 10 mL 0.5%  $\text{KH}_2\text{PO}_4$ , 2 mL 2.5M  $\text{MgCl}_2$ .) The protoplasts were washed once with PM and then resuspended in 3 mL of the same buffer.

Transformation was accomplished by centrifuging 200  $\mu\text{L}$  of protoplasts for 15 seconds in a microfuge, decanting the supernatant, and resuspending the protoplasts in the PM remaining in the tube. Ten  $\mu\text{L}$  of DNA solution was added (3  $\mu\text{L}$  of pEryAT1/rapAT14 DNA from Example 14 at about 1  $\mu\text{g}/\mu\text{L}$  in 7  $\mu\text{L}$  of PM buffer) and mixed with the protoplasts by gently tapping the tube. Two tenths of a milliliter of 25% PEG 8000 in T buffer (Hopwood, et al, 1985, Genetic Manipulation of Streptomyces A Laboratory Manual, The John Innes Institute) was then added, mixed by pipetting the solution 3 times and the suspension immediately spread on a dried R3M plate. The plate was incubated at 30°C for 20 hours and overlaid with 2 mL of water containing 100  $\mu\text{g}/\text{mL}$  thiostrepton, dried briefly and incubated 4 more days at 30°C.

To select stable transformants (integrants) colonies arising on the transformation plates were re-streaked onto R3M plates containing thiostrepton (20  $\mu\text{g}/\text{mL}$ ). Four colonies were confirmed to be thiostrepton resistant and were inoculated into 30 mL of SGGP containing thiostrepton (10  $\mu\text{g}/\text{mL}$ ). After growth for 3 days, one mL of each culture was extracted with ethyl acetate as described in Example 5, and run on a TLC plate to confirm that the strains were no longer making erythromycin A due to insertional inactivation by the integrating plasmid.

Integrants #1 and #4 were grown in SGGP without antibiotic for four days and then plated onto non-selective R3M plates for sporulation. Spores were plated on R3M plates to obtain individual colonies, which were then screened for sensitivity to thiostrepton, indicating loss of the plasmid sequence from the chromosome. Six thiostrepton sensitive colonies were

isolated from integrant #4 and one of these (4-A-1) was confirmed by Southern hybridization to have the EryAT1 replaced by the rapAT14 (FIG. 25). Hybridization was at 65°C and the stringency wash was with 0.1x SSC at 65°C. The strain was named *Sac. erythraea* ER720 EryAT1/rapAT14.

**EXAMPLE 16: Analysis of compounds produced by  
*Sac. erythraea* ER720 EryAT1/rapAT14**

Compounds produced by the recombinant *Sac. erythraea* strain, ER720 EryAT1/rapAT14, whose construction is described in Example 15, were characterized by TLC and mass spectrometry. For TLC analysis strain 4-A-1 was grown in SCM medium (20 g Soytone, 15 g Soluble Starch, 10.5 g MOPS, 1.5 g Yeast Extract and 0.1 g CaCl<sub>2</sub> per liter of distilled H<sub>2</sub>O) for 4 days at 30° C. The culture (1.5 mL) was centrifuged for 1 minute in a microfuge to remove cells. One mL of the resulting supernatant was removed to another microfuge tube and the pH adjusted to 9 by the addition of 6 µL of NH<sub>4</sub>OH. Then 0.5 mL of ethyl acetate was added, the tube was vortexed for 10 sec and then centrifuged for approximately 5 min to achieve phase separation. The organic phase was removed to another tube, and the aqueous phase was re-extracted with 0.5 mL of ethyl acetate. The second organic phase was combined with the first and dried in a Speed Vac. The residue was taken up in 13 µL of ethyl acetate and 10µL was spotted onto a Merck 60F-254 silica gel TLC plate. The plate was run in isopropyl ether:methanol:NH<sub>4</sub>OH (75:35:2). Erythromycin derivatives were visualized by spraying the plates with anisaldehyde:sulfuric acid:ethanol (1:1:9). Using this reagent, a novel compound predicted to be 12-desmethyl-12-deoxyerythromycin A, appeared as a blue spot running slightly faster than erythromycin A (FIG. 26).

To determine whether the novel spot seen on TLC has the molecular mass corresponding to the predicted 12-desmethyl-12-deoxyerythromycin A, an ethyl acetate extract was further analyzed by Mass Spectrometry. *Sac. erythraea* ER720 EryAT1/rapAT14 was grown in SCM medium for 4 days. Ten mL of culture was centrifuged to remove mycelia and pH of the supernatant was adjusted to 9 with NH<sub>4</sub>OH. The supernatant was then extracted twice with ethyl acetate and the organic phases pooled and dried. As shown in FIG. 33, mass spectrometric analysis of this crude ethyl acetate extract shows the mass of the novel spot to be 704, which corresponds to the molecular ion plus a proton (M+H<sup>+</sup>) of 12-desmethyl-12-deoxyerythromycin A.

**Example 17: Construction of plasmid pEryAT2/rapAT14**

pEryAT2/rapAT14 was constructed using standard methods of recombinant DNA technology according to the schematic outlines of FIGS. 15 and 34. To make a gene-replacement-vector specific for the *ery* AT2 domain, the two DNA regions immediately

adjacent to *ery* AT2 were cloned and positioned adjacent to the DNA encoding the *rap*AT14 domain in order to allow homologous recombination to occur. The strategy and protocol for constructing the intermediate plasmid containing the flanking regions, pCS5/AT2-flank, are described in Example 6 and FIG. 14. The final step in the construction of pEryAT2/*rap*AT14 was to ligate the 1 kb *rap*AT14-encoding DNA fragment having *Avr*II and *Nsi*I ends to pCS5/AT2-flank (Example 6) cut with the same enzymes to give the gene replacement/integration plasmid pEryAT2/*rap*AT14 (FIG. 27). All ligations were transformed into the intermediate host *E. coli* DH5 $\alpha$  and clones selected as previously described.

#### Example 18: Construction of *Sac. erythraea* ER720 EryAT2/*rap*AT14

A 10-desmethylerythromycin A and 10-desmethyl-12-deoxyerythromycin A producing microorganism was prepared by replacing the DNA fragment encoding the methylmalonyl acyltransferase domain of module 2 of the erythromycin PKS (EryAT2) of *Sac. erythraea* ER720 with a DNA fragment encoding a malonyl acyltransferase domain (*rap*AT14) from *S. hygroscopicus* ATCC 29253. This was accomplished with the recombinant plasmid, pEryAT2/*rap*AT14, prepared as described in Example 17. Transformation of ER720 and resolution of the integration event were carried out as described in Example 4 using 10  $\mu$ L of DNA solution consisting of 3  $\mu$ L of pEryAT2/*rap*AT14 DNA at about 1  $\mu$ g/ $\mu$ L in 7  $\mu$ L of P<sub>M</sub> buffer. One thiostrepton resistant colony was isolated and was inoculated into SGGP containing thiostrepton (10  $\mu$ g/mL) to isolate chromosomal DNA for Southern analysis. Integration of the plasmid DNA into the ER720 chromosome was further confirmed by Southern hybridization (data not shown). Hybridization was at 65°C and the stringency wash was with 0.1x SSC at 65°C.

The confirmed integrant was grown in SGGP without antibiotic for four days and then diluted 1000 fold into fresh medium and grown for 4 more days. Protoplasts were then prepared and plated onto non-selective R3M plates to obtain individual colonies, which were then screened for sensitivity to thiostrepton, indicating loss of the plasmid sequence from the chromosome. Four thiostrepton sensitive colonies were selected and 3 of these were confirmed by Southern hybridization, using conditions described above, to have the EryAT2 replaced by *rap*AT14 (FIG. 28). The strain was named *Sac. erythraea* ER720 EryAT2/*rap*AT14.

#### Example 19: Analysis of compounds produced by

#### *Sac. erythraea* ER720 EryAT2/*rap*AT14

Compounds produced by the recombinant *Sac. erythraea* strain, ER720 EryAT2/*rap*AT14, whose construction is described in Example 18, were characterized by TLC, bioassay, and mass spectrometry.

For TLC analysis cells were grown in either SGGP or SCM medium (20 g Soytone, 15 g Soluble Starch, 10.5 g MOPS, 1.5 g Yeast Extract and 0.1 g CaCl<sub>2</sub> per liter of distilled H<sub>2</sub>O) for 4-5 days at 30°C. Culture (22 mL) was centrifuged for 5 minute to remove cells. The resulting supernatant was removed to another tube and the pH adjusted to 9.0 by the addition of 6μL/mL of NH<sub>4</sub>OH. Then an equal volume of ethyl acetate was added, the liquid was mixed for 2 min. and then centrifuged for approximately 5 min. to achieve phase separation. The organic phase was removed to another tube, and the aqueous phase was re-extracted with half volume of ethyl acetate. The second organic phase was combined with the first and dried in a Speed Vac. The residue was taken up in 100 μL of ethyl acetate and one fourth of the sample was spotted onto a Merck 60F-254 silica gel TLC plate. The plate was run in isopropyl ether:methanol:NH<sub>4</sub>OH (75:35:2). Erythromycin derivatives were visualized by spraying the plates with anisaldehyde:sulfuric acid:ethanol (1:1:9). Using this reagent, two novel compounds predicted to be 10-desmethylethromycin A and 10-desmethyl-12-deoxyerythromycin A, appeared as blue spots with the lower spot running slightly slower than erythromycin A and upper spot running slightly faster than erythromycin A (FIG. 29).

To detect biological activity, a bioassay was performed. In this assay, another fourth of the extracted sample from above was spotted onto a disc. The disc was then air-dried and placed over a plate containing 50 mL of antibiotic medium 11 (DIFCO-BACTO) containing *Staphylococcus aureus* as an indicator strain. The inhibition zones were developed by overnight incubation of the plate at 37°C. As shown in FIG. 30, the novel compounds have bioactivity.

To determine whether the novel spots seen on TLC have the molecular mass corresponding to the predicted 10-desmethylethromycin A and 10-desmethyl-12-deoxyerythromycin A, an ethyl acetate extract from another culture was further analyzed by mass spectrometry. The sample was a crude extract of a 20 mL culture grown for 4 days. Mass spectrometric analysis revealed the two novel compounds to have masses of 720 and 704, which correspond to the molecular ion plus a proton (M+H<sup>+</sup>) of 10-desmethylethromycin A and 10-desmethyl-12-deoxyerythromycin A, respectively.

#### Example 20: Cloning of the ethylAT Domain from *Streptomyces caelestis*

A genomic library of *Streptomyces caelestis* NRRL-2821 (U.S. Patent 3,218,239 issued November 16, 1965) DNA was constructed in the bifunctional cosmid pNJ1 (Tuan, *et al.*, *Gene*, **90**: 21-29 (1990)). Cosmid vector was prepared by digesting 5 μg of pNJ1 with *Eco*R1, dephosphorylating with CIAP and then digesting with *Bgl*II to generate one arm and also digesting 5 μg of pNJ1 with *Hind*III, dephosphorylating with CIAP and then digesting with *Bgl*II to generate the other. Insert DNA was prepared by partially digesting approximately 5 μg of chromosomal *S. caelestis* NRRL-2821 DNA with *Sau*III according

to the procedure outlined in Maniatis *et al.*, *supra*. Digestion conditions were chosen which produced fragment sizes of approximately 40 kb. The ligation was performed by mixing approximately 1 µg of the digested chromosomal DNA with 0.5 µg of each cosmid arm. The ligation was incubated at 16°C overnight. GigapackII XL (Stratagene®) was used for packaging 2 µL of the ligation mix according the manufacturer's instructions. Transformation was done in *E. coli* XL1-Blue MR cells (Stratagene®). Individual colonies were picked into thirty 96-well plates to give a 99.99% probability that the library represented all *S. caelestis* NRRL-2821 genomic sequences.

The library was screened using a probe specific for the *S. caelestis* NRRL-2821 PKS region. The probe was generated by PCR amplification of *S. caelestis* NRRL-2821 genomic DNA using degenerate primers designed from consensus ketosynthase (KS) and acyltransferase (AT) sequences in the GenBank database. The KS specific oligo (SEQ ID NO:19) and the AT specific oligo (SEQ ID NO:20) generated a 900 bp PCR fragment. The PCR reaction contained 10 µL ThermoPol Buffer, 2 µL formamide, 25 µL of 20% glycerol, 3 µL 50 mM MgCl<sub>2</sub>, 45 µL water, 50 pmole of each primer, and approximately 0.2 µg DNA. The sample was heated to 99°C for 5 minutes, and then placed on ice, at which time a 10 µL cocktail consisting of 2 µL of a 10 mM mixture of dATP, dCTP, dGTP, and dTTP, 2 units of Vent DNA polymerase, and 7 µL of water was added. The sample was then transferred to a GeneAmp 9600 thermocycler (Perkin Elmer, Foster City, CA) and a temperature cycle of 1 minute at 95°C, 4 minutes at 50°C, and 4 minutes at 72°C was repeated 30 times, followed by a 15 minute incubation at 72°C. The desired PCR fragment was then isolated from 1.0% low melting agarose by standard procedures. The KS/AT probe was made by labeling approximately 50 ng of the PCR fragment with <sup>32</sup>P using the Megaprime DNA Labeling System (Amersham Life Science, Arlington Heights, IL). Library clones (2,880) were transferred from the 96-well plates to Hybond-N nylon filters (Amersham) and screened with the KS/AT probe according to procedures in Maniatis, *et al.*, *supra*. Hybridization was performed at 65°C and the final wash was in 0.1x SSC at 65°C. Nineteen of the clones hybridized strongly with the probe. These clones were then digested with *Sst*I, run on a 1.0% agarose gel and transferred to Hybond-N nylon filters for Southern analysis using the KS/AT probe (FIG. 31). The cosmid identified as pCEL18h5 was chosen for further analysis since it contained the largest number of hybridizing restriction fragments.

The *Sst*I fragments from cosmid pCEL18h5 were cloned into pGEM-3Zf (Promega, Madison, WI) and sequenced using the fmole DNA Cycle Sequencing System (Promega). The reactions were run on a Sequi-Gen II Sequencing Apparatus (Bio-Rad, Hercules, CA). Individual fragments were oriented relative to one another by sequencing off of cosmid pCEL18h5 using primers that hybridized to the 5' and 3' ends of the fragments to generate upstream and downstream sequence. These sequences were then matched with sequences from the individual fragments to place them in the proper order. A very large *Sst*I fragment



(>10 kb) was further digested with *Sma*I to generate smaller fragments for cloning and sequencing.

By searching the GenBank database with the sequences obtained it was possible to identify the various enzymatic motifs associated with the niddamycin PKS cluster and to group these motifs into modules (see FIG. 32) based on previous knowledge of Type I PKS organization. The C-6 position of the niddamycin macrolactone ring has an aldehyde derived from an ethyl side chain (FIG. 33). It was thus predicted that the AT of module 5 of the niddamycin cluster is responsible for incorporating this ethyl group into the growing chain. In addition, the carbon at C-7 of the molecule is completely saturated leading to the prediction that ER and DH motifs would also be present in module 5. These motifs were, in fact, found at the predicted region of the sequence. Furthermore, motifs for the preceding 4 modules were as predicted, with an inactive ketoreductase motif in module 4 which leaves a keto group at C-9 of the ring. Sequencing of that KR showed that the nucleotide binding site GXGXXG (SEQ ID NO:27) was mutated to DXTXXP (SEQ ID NO:28). The nucleotide sequence (SEQ ID NO:29) and corresponding amino acid sequence (SEQ ID NO:33) of the ethyl AT of module 5 are shown in FIG. 34 (top and bottom strands respectively).

A knockout experiment was also performed on this cluster, demonstrating that this sequence of DNA encodes the pathway for niddamycin biosynthesis (data not shown).

#### EXAMPLE 21: Construction of plasmid pEAT4

A multistep strategy was used to construct the plasmid pUC/ethAT/C6 (FIG. 35), which consists of the DNA encoding the NidAT5 domain flanked by approximately 2.0 kb of sequence upstream and downstream from the eryAT4 encoding sequences, all contained in pUC19. EryAT4 flanking DNA was subcloned from pAIBX85. This plasmid is a pCS5 derivative containing 8.4 kb of *Sac. erythraea* DNA from an *Xho*I site to a *Bam*HI site in the *eryAII* gene of the erythromycin PKS cluster. These sites correspond to bases 23211 and 31581, respectively, of GenBank accession number M63676. The EryAT4 5'-flanking DNA was isolated by digesting pAIBX85 with *Msc*I and *Bst*EII (corresponding to nucleotides 23,211 and 31,581, respectively). The resulting 1800 bp DNA fragment was treated with the Klenow Fragment of DNA Polymerase I, ligated into the *Sma*I site of pUC19, and transformed into *E. coli* DH5 $\alpha$ . Clones were selected on LB plates containing 150  $\mu$ g/mL ampicillin and 50  $\mu$ L of a 2% solution of X-gal for blue/white selection. The clones were confirmed by restriction analysis, resulting in the intermediate vector pUC/5'-flank. For convenient cloning of the NidAT5-encoding sequences, an *Avr*II site was engineered at the 3' end of the 5' flanking DNA. This was accomplished by PCR amplification from the *Pml*I site of the 5' flanking DNA to the *Bst*EII site with two oligonucleotides (SEQ ID NO:21 and SEQ ID NO:22). SEQ ID NO:22 incorporates an *Avr*II site and a *Bam*HI site at the 3' end of the 5' flanking DNA. PCR conditions were as described in Example 20 using *Sac. erythraea* DNA

as template with the following changes: Taq polymerase (GIBCO BRL) was used with the accompanying 10x buffer instead of Vent<sub>R</sub>® DNA polymerase and cycling conditions were 96°C/30 sec, 55°C/30 sec, 72°C/30 sec for 25 cycles. The resulting 300 bp PCR fragment was then digested with *Pml*I and *Bam*HI, gel purified from a 1.0 % agarose gel with Prep-A-Gene, and ligated back into pUC/5'-flank digested with *Pml*I and *Bam*HI to give pUC/5'-flank-*Avr*II. The ligation was transformed into DH5α and plated onto LB plates containing 150 μg/mL ampicillin. Clones were confirmed by restriction analysis and DNA sequencing.

In order to clone the NidAT5-encoding DNA fragment downstream of the 5' flanking DNA, an *Avr*II site was also engineered at the 5' end of the NidAT5-encoding DNA. As depicted in FIG. 36, an *Avr*II site could be engineered into the NidAT5 DNA without altering the amino acid sequence. Two PCR oligonucleotides (SEQ ID NO:23 and SEQ ID NO:24) were designed to create an *Avr*II site at the 5' end and a *Bam*HI site at the 3' end, respectively, of the NidAT5-encoding DNA. A convenient *Fse*I site occurs naturally at the 3' end of NidAT5-encoding sequence, so the resulting PCR fragment contains an *Fse*I site just upstream of the PCR engineered *Bam*HI site. SEQ ID NO:23 and SEQ ID NO:24 were used in a PCR reaction with the template p16-2.2. This plasmid is pUC19 containing a 2.2 kb *Sma*I fragment from module 5 of the niddamycin PKS cluster (see FIG. 32), which encompasses the sequences encoding NidAT5. The resulting 1.0 kb PCR fragment was digested with *Avr*II and *Bam*HI, purified from a 1.0 % agarose gel using Prep-A-Gene, and cloned into the *Avr*II/*Bam*HI sites of pUC/5'-flank-*Avr*II. Clones were confirmed by restriction analysis and DNA sequencing, creating the intermediate plasmid pUC/5'-flank/ethAT.

The EryAT4 3' -flanking DNA was subcloned by digesting pAIBX85 with *Pml*I and *Msc*I, corresponding to nucleotides 29,231 and 31,209, respectively, from the *eryAII* gene (GenBank accession number M63676). The DNA was gel purified on a 1.0 % agarose gel using Prep-A-Gene and ligated into the *Sma*I site of pUC19. The ligation was transformed into DH5α and plated as described previously. Clones were confirmed by restriction analysis, resulting in the plasmid pUC/3'-flank.

Attachment of the EryAT4 3'-flanking DNA to the NidAT5 -encoding sequence was accomplished by digesting plasmid pUC/3'-flank with *Fse*I and *Bam*HI, gel purifying the fragment from a 1.0 % agarose gel using Prep-A-Gene, and ligating it into pUC/5'-flank/ethAT that had been previously digested with *Fse*I and *Bam*HI. The ligation was transformed into DH5α as before and clones were analyzed by restriction analysis, resulting in the intermediate plasmid pUC/ethAT/C-6. The final step was to remove the NidAT5/flanking DNA insert from pUC/ethAT/C-6 with *Eco*RI and *Hind*III and ligate it into the *Eco*RI/*Hind*III sites of pCS5, resulting in the gene replacement/integration plasmid pEAT4 (FIG. 37).

EXAMPLE 22: Construction of *Sac. erythraea* ER720 EAT4-46

An example of a 6-desmethyl-6-ethylerythromycin A producing microorganism was prepared by replacing the DNA fragment encoding the methylmalonyl acyltransferase domain in module 4 of the erythromycin PKS (EryAT4) of *Sac. erythraea* ER720 with a newly discovered DNA fragment encoding an ethylmalonyl acyltransferase domain (NidAT5) from *S. caelestis* NRRL-2821. This was accomplished using the recombinant plasmid pEAT4, prepared as described in Example 21. Transformation of *Sac. erythraea* ER720 and resolution of the integration event were carried out according to the procedures described in Example 4 using 10  $\mu$ L of a DNA solution consisting of 3  $\mu$ L of pEAT4 DNA from Example 21 at about 1  $\mu$ g/ $\mu$ L in 7  $\mu$ L of PM buffer. One colony was confirmed to be thiostrepton resistant and was inoculated into SGGP containing thiostrepton (10  $\mu$ g/mL) to isolate chromosomal DNA for Southern analysis. Integration of the plasmid DNA into *Sac. erythraea* ER720 was confirmed by Southern analysis (data not shown). Hybridization was at 65°C and the stringency wash was with 0.1x SSC at 65°C.

The confirmed integrant was then subcultured into 30 mL SGGP without antibiotic using 10  $\mu$ L of the previous culture. After three days growth at 30°C the strain was again subcultured into 30 mL of fresh SGGP as before and plated onto non-selective R3M plates for sporulation. Spores were plated on R3M plates to obtain individual colonies, which were then screened for sensitivity to thiostrepton, indicating loss of the plasmid sequence from the chromosome. Nine thiostrepton sensitive colonies were isolated and three of them were confirmed by Southern hybridization to have the EryAT4 replaced by NidAT5 (FIG. 38). Hybridization was at 65°C and the stringency wash was with 0.1x SSC at 65°C. The strain was named *Sac. erythraea* ER720 EAT4-46, referred to as simply EAT4-46.

EXAMPLE 23: Analysis of compounds produced by EAT4-46

Compounds produced by strain EAT4-46, whose construction is described in Example 22, were characterized by TLC, bioautography and mass spectrometry.

The cells were grown in 30 mL of SCM for 4-5 days at 30°C. The culture was centrifuged for 10 minutes in a Sorval GLC-4 General Laboratory Centrifuge at setting 10 to remove cells. The resulting supernatant was adjusted to pH 9.0 by the addition of 180  $\mu$ L of NH<sub>4</sub>OH. Then 15 ml of ethyl acetate was added, the tube was vortexed for 30 seconds and then centrifuged for 10 minutes to achieve phase separation. The organic phase was removed to another tube, and the aqueous phase was re-extracted with 15 ml of ethyl acetate. The second organic phase was combined with the first and dried in a Speed-Vac. The residue was taken up in 30  $\mu$ L of ethyl acetate and 10  $\mu$ L was spotted onto a Merck 60F-254 silica gel TLC plate. The plate was run in a solvent containing isopropyl ether:methanol:NH<sub>4</sub>OH (75:35:2). Erythromycin derivatives were visualized by spraying the plates with anisaldehyde:sulfuric acid:ethanol (1:1:9). The results showed that EAT4-46 produced a

compound that migrated with the same *r<sub>f</sub>* as erythromycin A produced by wild type *Sac. erythraea* ER720, except in much lower yield (data not shown).

To determine the molecular mass of the compound, an ethyl acetate extract was prepared from a 50 mL SCM culture of EAT4-46 as described above, using a proportionate amount of reagents. The resulting residue was taken up in 50  $\mu$ L of ethyl acetate and run on a TLC plate as described previously, except that the plate was not sprayed with anisaldehyde. The compound of interest was isolated by scraping the silica resin in the vicinity of the spot and extracting the resin as described in Example 8. Mass spectrometric analysis revealed that the compound produced by the EAT4-46 strain had a mass of 734, which corresponds to the molecular ion plus a proton ( $M+H^+$ ) of erythromycin A.

In an attempt to increase substrate pools for the NidAT5 ethylmalonyl AT construction, the EAT4-46 strain was grown in 100 mL of SCM media containing 50 mM butyric acid, pH 7.0. The culture was grown for 4 days at 30°C and then centrifuged for 10 minutes in a Sorval GLC-4 Centrifuge to pellet the cells. The resulting supernatant was adjusted to pH 9.0 by the addition of 600  $\mu$ L of  $NH_4OH$  and extracted twice with 1/2 volumes of ethyl acetate as described previously. After drying in a Speed-Vac rotary concentrator, the extracted material was taken up in 100  $\mu$ L of ethyl acetate and 10  $\mu$ L was used for TLC analysis as described previously. Two spots running near *eryA* were observed in the butyric acid fed culture as opposed to only one spot in SCM media alone (FIG. 39). To determine the molecular mass of the two spots, most of the remainder of the extract was again subjected to TLC, and the compounds in the *eryA* region of the plate were isolated as described previously. Mass spectrometric analysis revealed that the two spots had molecular masses of 734 and 748. A molecular mass of 734 corresponds to the molecular ion plus a proton ( $M+H^+$ ) of erythromycin A, whereas the species of molecular mass 748 is consistent with the molecular mass plus a proton ( $M+H^+$ ) of ethylerythromycin A.

#### EXAMPLE 24: Cloning of the NidAT6 Domain from *Streptomyces caelestis* NRRL-2821

A genomic library of *Streptomyces caelestis* NRRL-2821 DNA was generated and screened with a probe specific for PKS genes as described in Example 20. From Southern analysis of *Sst*I digests of the positive clones (FIG. 31), some clones were selected for further analysis. These clones were digested with *Sma*I and run on a 1% agarose gel for Southern hybridization with the PKS specific probe. The analysis revealed that a second cosmid, pCEL13f5, shared many hybridizing bands with pCEL18h5, but also contained two unique bands of 1.9 kb and 6.0 kb (FIG. 40). This cosmid was chosen for further analysis in order to determine the sequence of the remaining PKS genes in the niddamycin pathway. Cosmid pCEL13f5 was digested with *Sst*I and the fragments were ligated to pUC19. A large *Sst*I fragment (>10 kb) was further digested with *Sma*I and ligated to pUC19. The ligations were

transformed into DH5 $\alpha$  cells and clones were selected on LB plates containing 150  $\mu$ g/mL ampicillin and 50  $\mu$ l of a 2% solution of X-gal for blue/white selection. DNA from clones containing the appropriate insert was isolated using the QIAprep Spin Plasmid Kit (QIAGEN Inc., Chatsworth, CA). Subclones were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer), and the reactions were run on a 4.75% acrylamide, 8.3 M urea gel in an Applied Biosystems 373 DNA Sequencing System. Ordering of the inserts and motif identification was done as described in Example 20.

The insert in cosmid pCEL13f5 was found to be approximately 25 kb in length, and the 5' end of the insert had about 10 kb of identical sequence with the 3' end of the insert in pCEL18h5. Together, the two cosmids contain all of the PKS genes of the niddamycin pathway (FIG. 32). Based on the structure of niddamycin (FIG. 33), the AT contained in module 6 (NidAT6) may utilize hydroxymalonate (tartronate) in the biosynthesis of the C-3, C-4, and O-4 positions of the macrolactone ring of niddamycin. (S. Omura *et al.* (*J. Antibiotics* 36:611-613 (1983)) have suggested that glycolate may be incorporated in the biosynthesis of the C-3, C-4 and O-4 positions of leucomycin, a closely related 16-membered macrolide). The nucleotide sequence of NidAT6 (top strand, SEQ ID NO:30) and its corresponding amino acid sequence (lower strand, SEQ ID NO:34) are shown in FIG. 41. A comparison of the amino acid sequence of NidAT6 with other ATs in the Swissprot database shows that NidAT6 resembles methylmalonyl ATs (data not shown).

#### EXAMPLE 25: Construction of plasmid pUC18/NidAT6

Two PCR oligonucleotides (SEQ ID NO:25 and SEQ ID NO:26) are designed to subclone the 1024 bp DNA fragment encoding the NidAT6 domain from the niddamycin PKS cluster and to introduce two unique restriction sites, *AvrII* and *NsiI*, for cassette cloning. This necessitates nucleotide changes, shown in bold in FIG. 42, at the beginning and near the end of the NidAT6-encoding DNA sequence. The changes shown also cause the replacement of a proline codon near the N-terminus of the NidAT6 domain with a valine codon, in order to increase the similarity of the domain junction sequence to that found naturally for some of the AT domains of the rapamycin PKS. (In FIG. 42, the underlined nucleotides are the wild-type sequence.) In addition, two other restriction sites, *EcoRI* and *BglII*, are also introduced at the 5' ends of the N-terminal and C-terminal oligonucleotides, respectively, for convenient subcloning of the PCR-generated product. The approximately 1 kb NidAT6 domain encoding DNA is amplified using methods described in Reagents and General Methods from Cosmid pCEL13f5. The PCR product is digested with *EcoRI* and *BglII* and subcloned into the *EcoRI* and *BamHI* sites of pUC18. The ligation mixture is transformed into *E. coli* DH5 $\alpha$  (GIBCO BRL) according to the manufacturer's instructions and transformants are selected on LB plates containing 150  $\mu$ g/mL ampicillin and 50  $\mu$ L of a 2% solution of X-gal for blue/white selection. Clones are confirmed by restriction analysis and the fidelity of the

insert is confirmed by DNA sequencing. The final plasmid construct is named pUC18/NidAT6.

Example 26: Construction of plasmid pEryAT2/NidAT6

pEryAT2/NidAT6 is constructed using standard methods of recombinant DNA technology according to the schematic outlines of FIGS. 15 and 53. To make a gene-replacement-vector specific for the eryAT2 domain, the two DNA regions immediately adjacent to eryAT2 are cloned and positioned adjacent to the DNA encoding the NidAT6 domain in order to allow homologous recombination to occur. The strategy and protocol for constructing the intermediate plasmid containing the flanking regions, pCS5/AT2-flank, are described in Example 6 and FIG. 13. The final step in the construction of pEryAT2/NidAT6 is to ligate the 1 kb NidAT6-encoding DNA fragment having *AvrII* and *NsiI* ends to pCS5/AT2-flank (Example 6) cut with the same enzymes to give the gene replacement/integration plasmid pEryAT2/NidAT6 (FIG. 43). All ligation mixes are transformed into the intermediate host *E. coli* DH5 $\alpha$  and clones are selected and characterized as described previously.

Example 27: Construction of *Sac. erythraea* ER720 EryAT2/NidAT6

A 10-desmethyl-10-hydroxyerythromycin A and 12-deoxy-10-desmethyl-10-hydroxyerythromycin A producing microorganism is prepared by replacing the DNA fragment encoding the methylmalonyl acyltransferase domain of module 2 of the erythromycin PKS (EryAT2) of *Sac. erythraea* ER720 with a DNA fragment encoding a hydroxymalonyl acyltransferase domain (NidAT6) from *S. caelestis* NRRL-2821. This is accomplished with the recombinant plasmid, pEryAT2/NidAT6, prepared as described in Example 26. Transformation of ER720 and resolution of the integration event are carried out as described in Example 4 using 10  $\mu$ L of DNA solution consisting of 3  $\mu$ L of pEryAT2/NidAT6 DNA at about 1  $\mu$ g/ $\mu$ L in 7  $\mu$ L of P<sub>M</sub> buffer. Thiostrepton resistant colonies are isolated and inoculated into SGGP containing thiostrepton (10  $\mu$ g/mL) to isolate chromosomal DNA for Southern analysis. Integration of the plasmid DNA into the ER720 chromosome is further confirmed by Southern hybridization. Hybridization is at 65°C and the stringency wash is with 0.1x SSC at 65°C.

Confirmed integrants are grown in SGGP without antibiotic for four days and then diluted 1000-fold into fresh medium and grown for 4 more days. Protoplasts are then prepared and plated onto non-selective R3M plates to obtain individual colonies, which are screened for sensitivity to thiostrepton, indicating loss of the plasmid sequence from the chromosome. Thiostrepton sensitive colonies are then selected and these are confirmed by Southern hybridization, using conditions described above, to have the EryAT2 replaced by NidAT6. The strain is designated *Sac. erythraea* ER720 EryAT2/NidAT6.

Example 28: Analysis of compounds produced by  
*Sac. erythraea* ER720 EryAT2/NidAT6

Compounds produced by the recombinant *Sac. erythraea* strain, ER720

5 EryAT2/NidAT6, whose construction is described in Example 27, are characterized by TLC, bioassay, and mass spectrometry.

For TLC analysis cells are grown in either SGGP or SCM medium (20 g Soytone, 15 g Soluble Starch, 10.5 g MOPS, 1.5 g Yeast Extract and 0.1 g CaCl<sub>2</sub> per liter of distilled H<sub>2</sub>O) for 4-5 days at 30°C. The culture is centrifuged for 5 min. to remove cells. The  
10 resulting supernatant is removed to another tube and the pH adjusted to 9.0 by the addition of 6μL/mL of NH<sub>4</sub>OH. Then an equal volume of ethyl acetate is added, the liquid is mixed for 2 min. and then centrifuged for approximately 5 min. to achieve phase separation. The organic phase is removed to another tube, and the aqueous phase is re-extracted with a half volume of ethyl acetate. The second organic phase is combined with the first and dried in a  
15 Speed Vac. The residue is taken up in approximately 25 μL of ethyl acetate and 15 μL are spotted onto a Merck 60F-254 silica gel TLC plate. The plate is run in isopropyl ether:methanol:NH<sub>4</sub>OH (75:35:2). Erythromycin derivatives are visualized by spraying the plates with anisaldehyde:sulfuric acid:ethanol (1:1:9). Using this reagent, two novel compounds predicted to be 10-desmethyl-10-hydroxyerythromycin A and 12-deoxy-10-  
20 desmethyl-10-hydroxyerythromycin A, are expected to appear as blue spots running slightly slower than erythromycin A.

To determine whether the novel spots seen on TLC have the molecular mass corresponding to the predicted 10-desmethyl-10-hydroxyerythromycin A and 12-deoxy-10-desmethyl-10-hydroxyerythromycin A, the remaining extract is further analyzed by mass  
25 spectrometry. The two novel compounds are predicted to have masses of 736 and 720, which correspond to the molecular ion plus a proton (M+H<sup>+</sup>) of 10-desmethyl-10-hydroxyerythromycin A and 12-deoxy-10-desmethyl-10-hydroxyerythromycin A, respectively.

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